The influence of changing salinity and other seasonal factors on farmed oysters

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Table of Contents

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INTRODUCTION

The changing coastal and estuary environment

Marine bivalve molluscs populate shallow coastal waters and estuaries, where phytoplankton for feeding is most abundant (Dame 2012). In coastal waters and estuaries bivalves, such as oysters, have adapted to changing environmental conditions, including exposure to air, seasonal changes in ambient air and water temperature and variations in salinity (Dame 2012). Temperature and salinity are two of the dominant environmental factors which influence bivalve mollusc physiology (Shumway 1996; Kim and Powell 2009; Dame 2012). There are many additional seasonal changes, closely related to temperature and salinity, which also affect oyster physiology, including nutrient abundance, tidal patterns (Cheney et al. 2000), suspended sediment (Ropert et al. 2008) and oxygen saturation in the water (Shumway and Koehn 1982).

Temperature

Bivalve molluscs are poikilotherms and temperature controls the rate of many key physiological processes (Galtsoff 1964). For example, temperature is the major controlling factor for feeding, growth rate, (Chavez-Villalba et al. 2005; Dame 2012) respiration rate (Shumway and Koehn 1982), oxygen consumption (Dunphy et al. 2006; Dame 2012) and excretion, in marine bivalve molluscs (Dame 2012). Temperature also influences oyster haemocyte function and numbers (Samian and McCombie 2008). In triploid Pacific oysters (*C. gigas*), which were acclimatized to either 12 or 18^{o}C, there was corresponding acceleration in metabolism and higher haemocyte activity, including higher phagocytosis rate, greater synthesis of reactive oxygen species (ROS) by granulocyte haemocytes and increased numbers of circulating haemocytes as the ambient temperature increased (Samain and McCombie 2008). However in diploid Pacific oysters (*C. gigas*) once temperatures reached 21^{o}C there was a decrease in haemocyte phagocytic activity and numbers of circulating haemocytes (Samain and McCombie 2008). After incubation of haemocytes at 35-40^{o}C for four hours (similar to conditions for inter-tidal oysters out of water, in summer in France, where the research was conducted) there was degradation of haemocyte function including increased haemocyte mortality, and

lower haemocyte amino peptidase and esterase activity (Samain and McCombie 2008).

Salinity

Variation in salinity in the estuarine environment is another key environmental factor which affects a range of physiological processes and structural properties of bivalves, including oyster species (Shumway 1996, Dame 2012). Oysters are osmoconformers and changes in water salinity directly influence the osmotic concentrations of their extracellular fluids, the relative proportions of solutes in their extracellular fluids and the density and viscosity of extracellular fluids (Kinne 1964; Gullian and Aguirre-Macedo 2010). In addition, salinity affects the adsorption and saturation of dissolved gases in water (Dame 2012). By this means, salinity indirectly influences respiration rate (Dame 2012). The stress of abruptly changing salinity can directly affect bivalves by depressing their respiration for up to 2 days as they adapt and osmoconform to the new salinity (Berger and Kharazova 1997). Salinity can also directly and indirectly affect the immune system. Very low salinity (5ppt) and hyper salinity (41ppt) will suppress haemocyte phagocytosis rate (Gagnaire et al. 2006). Salinity will also indirectly influence the immune system. For example 32ppt favoured the proliferation and viability of *Listonella anguillarum* (a bacterial pathogen of *Ostrea edulis*) and there was corresponding increase in circulating granular haemocyte and haemolymph hydrogen peroxide concentration in *O. edulis* (Hauton et al. 2000). When the salinity dropped to levels unfavourable for *L. anguillarum* there was no appreciable rise in haemocyte numbers or haemolymph hydrogen peroxide concentrations compared to control oysters (Hauton et al. 2000).

In combination, temperature and salinity can often act synergistically on bivalve physiology (Kinne 1964; Dame 2012), including oysters (Galtsoff 1964). Together temperature and salinity directly affect processes governing ingestion, growth, filtration rate, respiration rate, oxygen consumption and the division of assimilation into somatic and gametic growth (Powell et al. 1992; Powell et al. 1994; Kobayashi et al. 1997; DiGialleonardo et al. 2005; La Peyre et al. 2009). For example, as temperature and salinity increase, gill activity increases resulting in a higher rate of filtration and ingestion of food (Powell et al. 1992). Temperature and salinity are two

factors which influence dissolved oxygen concentration in seawater uptake of dissolved oxygen by bivalve molluscs (Dame 2012) but their effects on oxygen consumption are not always equal. In the case of Eastern oysters (*C. virginica*), changing temperature influenced oxygen uptake more than changing salinity (Shumway and Koehn 1982). Temperature and rainfall (and subsequent changes in salinity) leading to nutrient input and phytoplankton production in coastal waters and estuaries, play a role in seasonal division or partitioning of somatic and gametic growth in preparation for spawning (Powell et al. 1992; De La Parra et al. 2005) .

Seasonal changes in temperature and salinity (which is often influenced by rainfall and river flows) will directly and indirectly, through phytoplankton availability for oyster nutrition, influence seasonal changes in oysters such as spawning (Westley 1964) . Phytoplankton (primary source of nutrition for oysters) concentrations in the water (which can also be measured indirectly by chlorophyll a concentrations (Soletchnick et al. 2006)) are determined by temperature and nutrient flows from rivers. These nutrient flows are enhanced by rainfall (Ropert et al. 2008). Temperature, salinity and phytoplankton levels influence the progression of leydig tissue production (glycogenesis), gametogenesis and finally spawning (De La Parra et al. 2005). The level of available phytoplankton contributes to the glycogen stores in oysters (Soniat et al. 1998). Glycogen production is closely related to the reproductive cycle (Hofmann et al. 1994) and once glycogen stores are maximised, gametogenesis begins in preparation for spawning (Soletchnick et al. 2006).

Significant variations in salinity and temperature in estuarine environments can also lead to a trade-off for oysters between growth rate, disease prevalence and predator numbers (Levinton et al. 2011). Influxes of freshwater can be beneficial to oysters by killing pests or predators such as gastropods, flatworms and starfish (Galtsoff 1964). Short periods of freshwater flushing (after high and intense rainfall events) and lower temperatures also limit infectious diseases such as Haplosporidiosis (Haskin and Ford 1982) and Perkinsiosis in Eastern oysters (Craig et al. 1989; Cook et al. 1998). The control of these parasites is often most evident in oysters which are resident at the head of a bay (closest to the river mouth) where the fall in salinity is greatest, rather than oysters closer to the mouth of the bay where the freshwater influx is

diluted by the tidal effect of the incoming sea water (Galtsoff 1964). Prolonged freshwater flooding will hinder the return of pathogens and predators for oysters but will also limit oyster growth because the oysters will remain closed longer and the phytoplankton load will be diluted (Levinton et al. 2011).

Oyster mortality events

The host – environment-pathogen interaction

Oyster mortality events can be grouped broadly in to a) non-infectious and b) infectious disease events. Mortality events due to non-infectious processes involve temporal and spatial correlations between external risk factors (e.g. environmental, nutritional or toxic factors), and host susceptibility factors (e.g. age) (Lower 1983) (Evans 1976). For example, multiple Eastern oyster *(Crassostrea virginica)* mortalities on the eastern and gulf coasts of the United States occurred after freshwater flooding in spring (environmental factors), when oysters had a higher metabolic rate and demand for glycogen production and later gamete production (all host factors) (Gunter 1950; Butler 1952; Shumway 1996). Sydney Rock oyster (*Saccostrea glomerata*) mortalities occurred after summer freshwater flooding with runoff of acid sulphate soils, and low water pH in the Hastings estuary NSW, Australia (Steen 1996). In both spring and summer oysters have higher metabolic rates and demands as they produce glycogen stores and later, gametes (Shumway 1996).

In the case of mortality events due to infectious processes, there are temporal and spatial correlations between infectious organisms or viruses with both external environmental risk factors and host susceptibility factors (Evans 1976; Lower 1983). For example Ostreid Herpesvirus 1 caused mortalities in Pacific oysters (*C. gigas*) larvae (age as host factor) during summer (environmental factor) in New Zealand (Hine et al. 1992). Differentiation between infectious and non-infectious causes of mortality is based on environmental monitoring, oyster mortality patterns (the correlation or lack of correlation between temporal and spatial mortalities events with environmental factors) and testing for the presence or absence of infectious pathogens in the affected and non-affected oysters, using tests such as

histopathology, microbiology or molecular biology (Cheney et al. 2000; Berthe 2008; Chaney and Gracey 2011).

Infectious causes of oyster mortalities

The interactions of infectious pathogens, environmental factors (which are either favourable for pathogen replication or cause stress in the oysters) and host oyster factors (such as age), associated with oyster mortalities are varied. For example in Tomales Bay, on the West coast of the United States when water temperatures rose above 20^{o}C (environmental risk factor) mortalities occurred most commonly among juvenile (host susceptibility factor) Pacific oysters (*C. gigas*) following Ostreid Herpesvirus 1 infection (Friedman et al. 2005). On the other hand, in France, mortalities of Pacific oyster (*C. gigas*) spat were associated with temperatures above 16 ^{o}C and infection with Ostreid Herpesvirus 1 micro variant, (Schikorski et al. 2011). On the east coast of the United States of America, the prevalence of *Haplosporidium nelsoni* infection in Eastern oysters (*C. virginica*), and the rate of mortalities increased as salinity increased (Haskin and Ford 1982). The prevalence of *Perkinsus marinus* infection, also in Eastern oysters, and rate of mortalities, increased as the water temperature increased (Cook et al. 1998). In Washington State, USA, and British Colombia, Canada, elevated water temperature along with increased nutrient levels were associated with outbreaks of Nocardiosis causing summer Pacific oyster (*C. gigas*) mortalities (Friedman 1990).

Non-infectious risk factors associated with oyster mortalities

Summer mortality syndromes

Although oysters adapt and respond to seasonal variations in temperature, salinity and nutrient in-put (De La Parra et al. 2005; Soletchnick et al. 2006) dramatic and sudden changes in multiple environmental factors, in the absence of evidence of infectious pathogens (using diagnostic tests available at the time of the investigation) are associated with oyster mortalities. The interaction of these multiple environmental stressors is often complex and it is generally the combined effect of these stress factors which are associated with oyster, such as Pacific oyster (*C. gigas*), mortalities (Cheney et al 2000, Berthe 2008, Chaney and Gracey 2011). For example, elevated summer temperatures, neap tides and associated oxygen

depleted water were risk factors for summer mortalities of Pacific oysters (*C. gigas*) in Puget Sound, Washington State, USA and pathogens were not identified by histopathology (Cheney et al. 2000; Cheney et al. 2001). In France, increasing water temperature (above 19^{o}C), high chlorophyll a levels , and intrinsic Pacific oyster (*C. gigas*) factors including well developed gonads, high glycogen condition and predisposing genetic susceptibility in oysters were risk factors for summer mortality. Surveys for Ostreid Herpesvirus 1 (OsHV1) by PCR were conducted during these mortality events in France but there was no statistical association between Pacific oyster (*C. gigas*) mortalities and OsHV1 prevalence, in the years up to 2008 (Samain and McCombie 2008). Elevated summer temperatures and high water nutrient loads influenced the onset of summer mortalities of Pacific oysters (*C. gigas*) at Bannow Bay and Dungarvan Harbour in Ireland and the Menai Strait and Inland Sea in Wales in 2003, however there was no record of diagnostic histopathology or molecular biology testing to exclude concurrent infectious pathogens (Malham et al. 2009). Summer mortality of Pacific oysters (*C. gigas*) in Matsushima Bay was associated with eutrophication and elevated summer temperatures (Mori 1979). On histopathology there were no bacterial, protozoal or micro-cell pathogens and fatty accumulations in digestive glands, an inefficient source of energy (compared to glycogen), was believed to have caused metabolic stress in pre-spawning oysters and subsequent mortalities (Mori 1979).

Flood events associated with oyster mortalities

Oyster mortalities associated with freshwater floods, are another example of environmental factors leading to oyster deaths. The effect of freshwater floods, and subsequent abrupt change to low salinity, on mortality rate in Eastern oysters (*C. virginica*) is highly dependent on ambient temperature (Galtsoff 1972). While estuarine oysters can tolerate freshwater during the winter, abrupt changes to very low salinities (5-10ppt) cause physiological stress under spring and summer temperature conditions because they coincide with increased metabolic demand of leydig tissue proliferation and gonad development prior to spawning (Shumway 1996). Specific areas in the USA where oyster mortalities occurred following spring or summer floods included the mouth of the Mississippi river (Gunter 1950; Butler 1952), upper reaches of Chesapeake Bay (Engle 1946; Andrews et al. 1959),

Santee River in South Carolina (Burrell 1977) and areas of Louisiana (Dugas and Perret 1973). Furthermore, freshwater floods were associated with Pearl oyster (*Pinctada imbricata*) mortalities at Port Macquarie on the north coast of NSW, Australia (O'Connor and Lawler 2004), and Pacific oyster (*C. gigas*) mortalities on Atlantic coast of France (Bodoy et al. 1990).

Freshwater floods consist of multiple factors. Along with temperature and abrupt fall in salinity, a range of substances transported in the freshwater, such as sediment, excess nutrients, for example phosphates and nitrates (which can lead to eutrophication), pesticides, hydrocarbons, and heavy metals are potential additional stress factors contributing to oyster deaths (Ropert et al. 2008). For example, the combination of high temperature, abrupt low salinity and eutrophication, after freshwater flooding in summer, contributed to Pacific oyster (*C. gigas*) mortalities in Geoje Bay, South Korea (Cho and Kim 1977). Eutrophication can occur after freshwater flooding, when excess nutrients and organic matter flow into estuarine or coastal seawater. The excess nutrients can result in toxic algal blooms and dramatic falls in dissolved oxygen leading to hypoxic injury to oysters and oyster mortalities (Guillaud et al. 1992; Gray et al. 2002; Van Beusekom and De Jonge 2002). Water catchment disturbances such as farming, fertilizer application and discharge of wastewater will increase the risk of eutrophication associated with freshwater flooding (Wu and Levings 1980; Paterson et al. 2003).

Mortalities in Tasmanian farmed oysters

In late January 2004 (summer) there was an unseasonal massive flood event (the largest flood since records began in 1968) into Georges and Moulting Bays, on the east coast of Tasmania and just 8 days later, significant Pacific oyster (*C. gigas*) mortalities (up to 90% on some leases) occurred (DPIWE Tasmania 2004a). Following the flood event there were prolonged low salinity conditions in the surface water. The farmed Pacific oyster (*C. gigas*) mortalities in the Georges and Moulting Bay MFDP (Marine Farm Development Plan) area were restricted to intertidal marine farming zones in western Georges Bay and Moulting Bay, which were closest to the river mouth. On the farmed leases pesticides and ammonia was not elevated. At one lease the concentration of dissolved zinc was raised in one of three serial samples

from that lease. All other leases had low or undetectable concentrations of zinc. Because there was only one elevated zinc result this may have been an anomaly and could not be conclusively correlated to the mortalities. There were detectable rises in concentrations of dissolved aluminium, iron, magnesium and manganese but the concentrations were well below toxic levels. The histopathological findings in oysters from oysters across Moulting and Georges Bay were consistent with environmental stressors, including osmoregulatory changes(expanded intercellular spaces in the renal and alimentary tissue) and subsequent mobilisation of energy reserves and necrosis associated with gastrointestinal insults (haemocyte infiltrate into the alimentary tract, and leydig tissue breakdown and necrosis) (DPIWE Tasmania 2004a). Freshwater flooding and oyster mortalities also occurred at the same time in Great Oyster Bay MFDP oyster leases (which are also on the east coast of Tasmania, approximately 150km south of Georges and Moulting Bays) but extensive investigation and sampling of oysters and water, similar to Georges and Moulting Bays, was not completed (DPIWE Tasmania 2004a).

Research objectives

This thesis will not attempt to explain in depth the processes for mortality investigation because this has been done extensively (Cheney et al. 2000; Samain and McCombie 2008; Chaney and Gracey 2011). This work will focus on the influence of environmental risk factors for farmed Pacific oysters and their host response. Where infectious diseases were investigated these methods and results will be outlined.

The objectives of the first part of the project were to undertake a retrospective study of the histopathological slides from Pacific oysters sampled during the Georges and Moulting Bay oyster mortality event of February 2004 and compare the histopathological changes to Pacific oysters in February 2003 and October 2005, from similar leases which were not part of a mortality event but were sampled at part of the Tasmanian Pacific Oyster Health Surveillance Program.

The second project then explored the capacity of oysters to adjust to one environmental stressor associated with freshwater floods, abrupt fall to low salinity,

by describing the histopathological changes, and interpreting these in conjunction with biochemical changes in the oyster. The influences of grading, family line and season on oysters' response to abrupt change to low salinity were observed. Describing the histopathological changes due to low salinity, a common environmental stressors, will aid interpretation of molluscan histopathology for diagnosticians.

This retrospective study was based on the research proposals from the report "Georges Bay Oyster ill thrift & mortality: DPIWE AH&W comments on future investigation".

Chapter 1

Oyster stock losses on estuarine farms during summer freshwater flooding: A 3 year retrospective study

INTRODUCTION

Tasmanian Pacific oyster (*Crassostrea gigas*) growers have not suffered significant industry-wide stock losses such as those experienced on the East coast of the United States (Chaney and Gracey 2011) and the Atlantic coast of France (Soletchnik et al. 2005; Schikorski et al. 2011) . However, in February 2004 following summer freshwater flooding there was a Pacific oyster (*Crassostrea gigas*) mortality event reported by oyster farmers in the Georges Bay / Moulting Bay Marine Farming Development Plan (MFDP) area on the east coast of Tasmania (Figure 1). The oysters ranged in size from 30 to 90 mm.

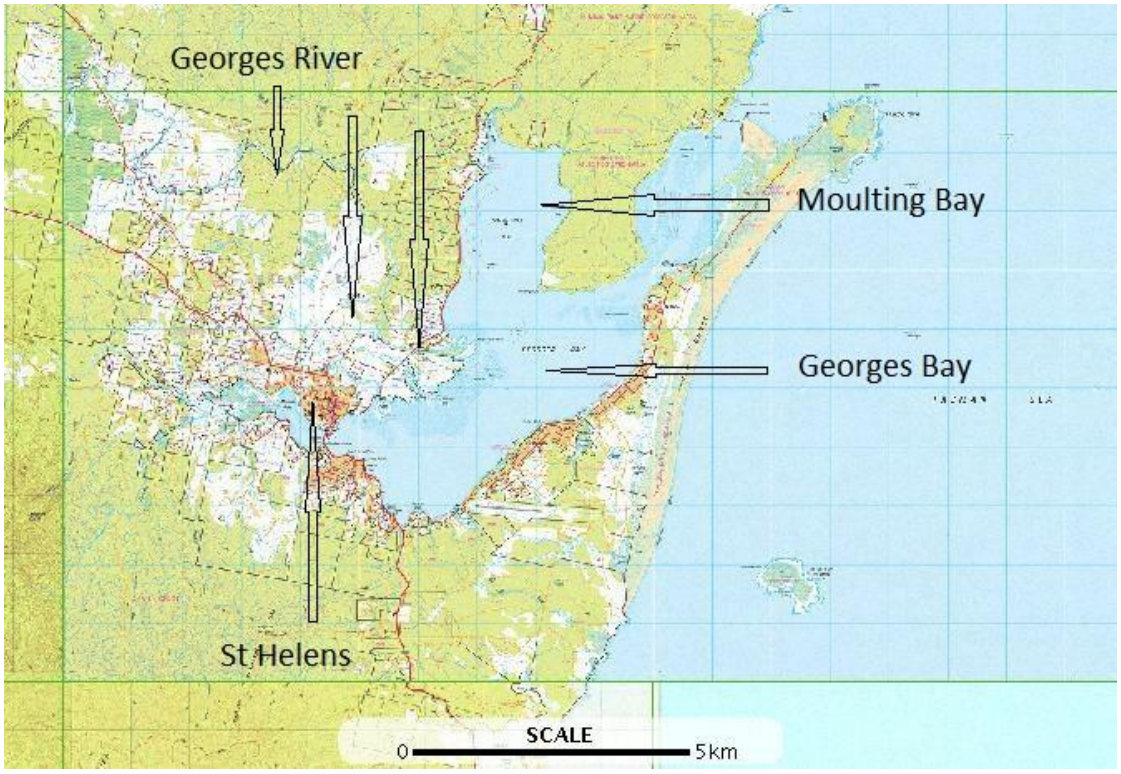

Figure 1: George River Catchment in north-eastern Tasmania (source: TASMAP, https://www.tasmap.tas.gov.au/do/product/25000TOPO/6042, accessed 18/9/13)

The flooding was due to the highest rainfall event in the George River catchment since records started in 1968. The George River flows into Georges and Moulting Bays near the town of St Helens. Pacific oyster mortalities (up to 90% on some leases) were localised to intertidal leases in western Moulting Bay and Georges Bay, nearest to the mouth of the George River, and no significant mortalities were observed in stock held on sub-tidal or intertidal leases in eastern Georges Bay near

the Barway, closest to the ocean (DPIWE Tasmania 2004a). Mortalities resolved after a period of approximately 10 days, when salinity returned to levels at 28 parts per thousand (ppt) (DPIWE Tasmania 2004a).

There are multiple pluviometric risk factors, along with the change in salinity, associated with freshwater input and in particular freshwater flooding (Ropert et al. 2008). Anthropogenic freshwater factors that could have contributed to oyster mortalities in the Georges and Moulting Bays, included acid sulphate soil run off (precipitated by land clearing), pesticides, herbicides, heavy metals, eutrophication and petroleum products or hydrocarbons (Percival and Ellard 2004). Other factors, unrelated to human endeavours, included increased suspended sediment concentration and low dissolved oxygen unrelated to eutrophication (Ropert et al 2008). Secondary bacterial infections (such as vibriosis) in oysters, challenged or stressed by freshwater flooded estuaries, could potentially also have contributed to on-going stock losses (Percival and Ellard 2004).

Because of the complexity of multiple potential stress factors, extensive water data records, which were measured during the flood event of February 2004 in Georges and Moulting Bay, were reviewed. These archived results were then compared with pre and post flood event water data records, to assess how the freshwater flood differed from the normal water environment. Many of the parameters measured during the floods were monitored at regular intervals throughout the year by the Tasmanian Shellfish Quality Assurance Program (TSQAP). These included pesticides, herbicides, heavy metals (in both the water and oyster meat) concentration of algal species, salinity and water temperature. This extensive testing is undertaken by TSQAP to maintain the product integrity of Tasmania oysters for human consumption (TSQAP www.dhhs.tas.gov.au, accessed 31/8/11).

Histopathological surveys of populations of oysters and other marine bivalve mollusc (such as mussel) are important components of bio-monitoring studies and are useful for understanding the response of oysters and mussels to environmental stressors (Yevich and Yevich 1994; Kim and Powell 2009). This study was a retrospective assessment of the freshwater flooding and oyster mortality event in February 2004,

in Georges and Moulting Bay. Histopathological slides were the most easily accessible archived material for assessing and comparing effects of environmental factors, associated with flood waters, on the oysters and identifying oysters' response to potential flood water stressors because extensive histopathological material was collected from oysters during the flood event. In addition, archived material was available at the Animal Health Laboratory from before and after the flood event from the Georges and Moulting Bay oyster leases. This material was part of the Tasmanian Pacific Oyster Health Surveillance Program, an annual program supported by the Tasmanian Oyster Research Council and oyster farmers, to monitor for infectious diseases which are exotic to Tasmania. The diseases surveyed include Iridovirus, Herpesvirus, Marteiliosis, Haplosporidiosis, Perkinsiosis, Nocardiosis, Mikrocytosis and the endemic infectious disease Bonamiosis, which is only found to cause disease in native Tasmanian flat oysters (*Ostrea angasi*) but which can potentially be carried in Pacific Oysters. Records and histopathological slides from February 2003, before the flood event, during the flood event in February 2004 and after the flood event in October / November 2005 were reviewed.

The specific objectives of this research were:
To determine if the effect of freshwater stress factors in February 2004 (in particular, abrupt change in low salinity) were reflected in histopathological findings in oysters, most importantly those on high mortality leases; and compare these histopathological changes to oysters before and after the flood event.

METHOD

Lease descriptions

Seven commercial oyster leases were included in this investigation. The oysters were grown in baskets suspended on lines. The leases were identified by letters (in order to keep the identity of the leases anonymous) and described by their a) type (inter-tidal / sub-tidal) and b) location in Georges and Moulting bays (Figure 2).

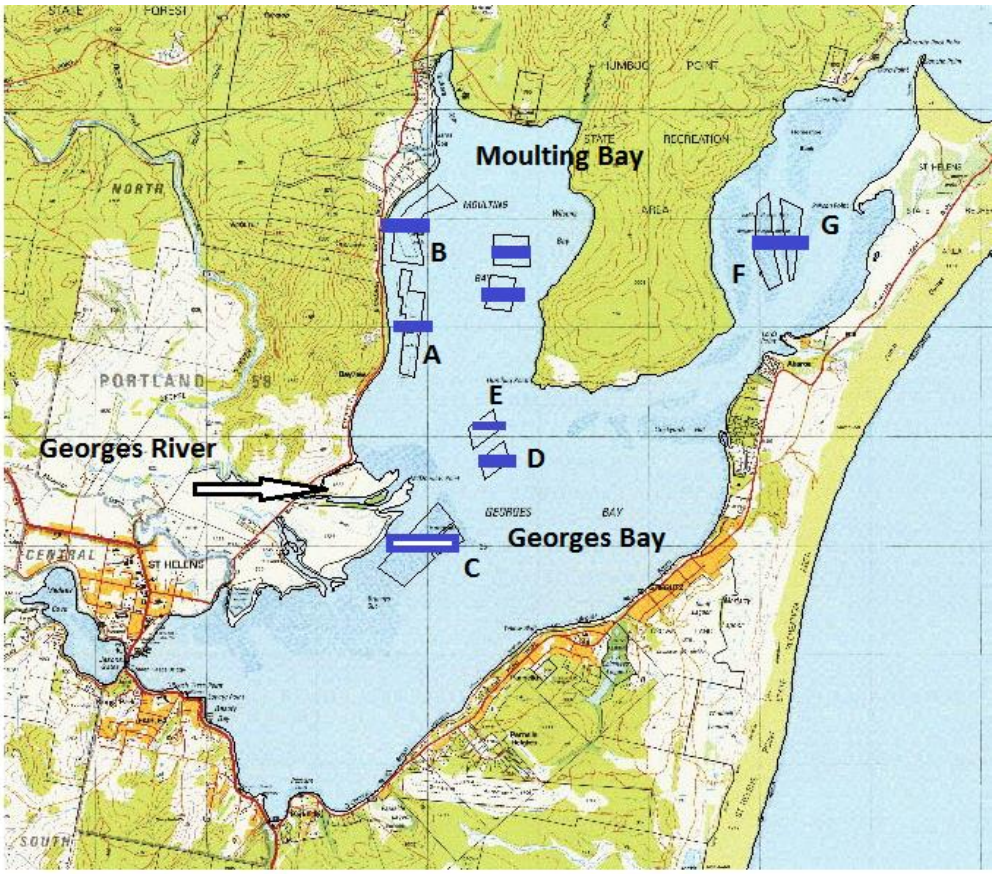

Figure 2: Location of oyster leases sampled in Georges Bay and Moulting Bay (Map from "Oyster Health in Georges Bay, collation and analysis of historical data" Percival and Ellard 2004). The lease numbers have been deleted with blue lines to maintain the anonymity of the leaseholders.

Leases A to C were inter-tidal leases in western Moulting and Georges Bays. Leases D and E were sub-tidal leases in central Georges Bay. Leases F and G were inter-tidal leases in eastern Georges Bay and were the closest leases to the Barway at the mouth of the bay leading to the sea (see Table 1).

Lease	Location
A B C	Western Moulting and Georges Bays
D E	Central Georges Bay
F G	Eastern Georges bay, near the mouth of the bay, closest to the sea

Table 1: Leases and their location in Georges and Moulting bays

Case histories, gross reports and microbiology results

All cases used in this study were from oyster farms in Georges Bay and Moulting Bay MFDP (Marine Farming Development Plan) area. Case histories from oyster farmers and gross descriptions by veterinary officers of DPIWE Tasmania (Dept. Primary Industries, Water and Environment) were accessed from archives at the Animal Health Laboratory Mt Pleasant, Tasmania, on the Laboratory Information Management System (LIMS). For this retrospective study records came from

a) February 2003 lease C and D as part of the annual TPOHSP

b) February 2004 leases A-F as part of the February mortality event investigation

c) October to November 2005; leases A-G as part of the TPOHSP

The time line of events and sampling times is recorded in Table 2.

Date	Rainfall event	Oyster sampling by DPIWE	Water sampling by DPIWE for water quality testing	Water quality sampling by TASQAP	Water sampling by DPIWE for algal tests by Phycotech	Mortality counts
2/02/2003				All leases		
18/02/2003		Lease C				
11/02/2003				All leases		
26/02/2003		Lease D				
27/01/2004 to 30/01/2004	Rainfall (226mm) and flood event					
31/01/2004 to 4/2/2004						
5/02/2004	DPIWE advised of mortality event	Leases A, B, C,E, F and G	Leases A-G and George river mouth		Leases A, B, C, D and G and mouth George River	
6/02/2004						
7/02/2004					Leases A, B, C, D and G the mouth of the George River	
8/02/2004						Lease B
9/02/2004						Lease B
10/02/2004						Lease B
11/02/2004						Lease B
12/02/2004			Leases B, C			Leases A, B, C and D
13/02/2004		Leases B, D and F	Lease A and D			

Table 2: Chronological chart of events and samples taken during February 2003, February 2004 and October to November 2005

Date	Rainfall event	Oyster sampling by DPIWE	Water sampling by DPIWE for water quality testing	Water quality sampling by TASQAP	Water sampling by DPIWE for algal tests by Phycotech	Mortality counts
10/10/2005		Leases A-F				
12/10/2005				All leases in Georges and Moulting Bays		
17/10/2005		Leases A-F				
21-22/10/2005	129.8mm over 48hrs					
24/10/2005		Leases A-F				
1/11/2005		Leases A-F				
3/11/2005				All leases in Georges and Moulting Bays		

Table 2 continued

The laboratory records:

a) Mortality rates were estimated on leases by sampling 10-11 random representative units (double basket, mesh bags or mesh envelopes) across each lease and counting the numbers of live and dead oysters. The mortality rate for each lease was the average mortality across all units (DPIWE 2004a).

b) Gross characteristics of the shell and meat of the oysters. These descriptions included abnormal conformation / shape of the shell (e.g. abnormal fluting), significant defects, which affected the integrity of the shell and the shell seal, and the colour and distribution of any other abnormalities. Gross lesions in the oyster meat

were described including the colour, distribution / pattern, shape / contour, size, organ or site and change in texture.

c) Microbiology; Oyster haemolymph was sampled from a subset of oysters during the February 2004 flood event. Haemolymph was sampled from 6 oysters, from leases E, F and G and 10 oysters from lease C. Additionally oyster meat was sampled from 4 dead oysters from lease C and a shell abscess from lease E. The haemolymph and meat were cultured on TBC and sorbitol SBA plates at the Animal Health Laboratory, Mt Pleasant, DPIPWE Tasmania.

Environmental records

Environmental records and water data (such as water salinity, water temperature, thermo tolerant coliform counts, and algal counts) for all commercial oyster leases in Tasmania are archived by the Tasmanian Shellfish Quality Assurance Program (TSQAP), Department of Human Health and Human Services Tasmania. For this study, records for the periods February 2003, February 2004 and October to November 2005 were obtained. Thermo-tolerant coliforms (colony forming units / 100ml) were conducted by Tasmanian Laboratory Services (Launceston Tasmania) for the TSQAP. Algal counts were conducted by the analytical laboratory Phycotech Pty Ltd, Hobart Tasmania for the TSQAP. In addition, near leases F and G (zone 6A) there was permanently anchored automated water testing devices recording continuous surface water salinity and temperature (data provided by TSQAP) (See Figure 3).

There were additional environmental water data records for 5th, 7th and 12th February 2004 during the flood event. Water quality analysis was undertaken by Analytical Services Tasmania, University of Tasmania, for a wide range of chemicals and elements (Table 3). Oyster meat was tested for tributyl tin, also at Analytical Services Tasmania. All these records were published in the “DPIWE Final Report – Oyster Mortalities in the Georges Bay Marine Farming Development Plan Area, February 2004” (DPIWE Tasmania 2004a).

Analyte	Test method
pH in Water	APHA Method 4500-H
Conductivity	APHA Method 2510
Total Dissolved solids	APHA Method 2540C
Alkalinity	APHA Method 2320/4500-CO2
Anions	Ion Chromatography APHA Method 4110B
Hardness	APHA Method 2340
Dissolved Nutrients	APHA Method 4500
Metals in Water	APHA Method 3030/3120
Major Cations in Water	APHA Method 3030/3120
Alkyl tin Compounds in Water	GCMS
Semi volatile Organics (pesticides) in Water	GCMS
Pesticides in Biota, Water & Soil	HPLC
Alkyl tin Compounds in Biota	GCMS
Pesticides in Biota	GCMS
Pesticides in Biota	HPLC
Semi volatile Organics in Biota - OC & OP Pesticides	GCMS

Table 3: List of analytical tests for water (DPIWE 2004a); the tests, calibrations or measurements were performed in accordance with NATA requirements which include the requirements of ISO/IEC 17025 and were traceable to national standards of measurement. (APHA = American Public Health Association, GCMS = Gas Chromatography and Mass Spectrometry, HPLC = High Performance Liquid Chromatography).

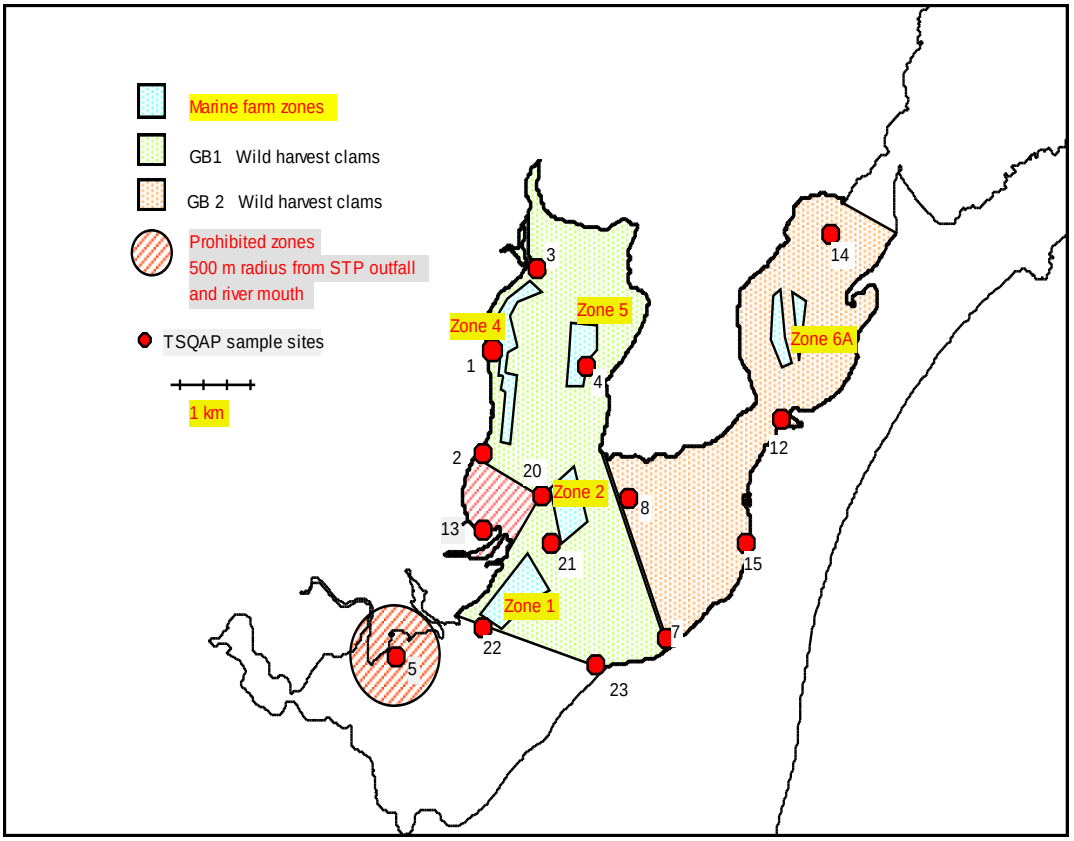

Figure 3: Sites for TSQAP water sampling and zones for leases (Source, TSQAP). Zone 1 includes inter-tidal lease C; Zone 2 sub-tidal leases D and E; Zone 4 inter-tidal leases A and B; and Zone 6 inter-tidal leases F and G; site 5 is the effluent outflow and site 13 George River. The data logger was anchored at zone 6A

The monthly rainfall data for St Helens aerodrome (the closet rainfall measurement site to Georges and Moulting bays) was obtained from the Bureau of Meteorology, Australian Government website: http://www.bom.gov.au/climate/data/index.shtml (accessed on 31/8/11).

Histopathology

Archived haematoxylin and eosin stained slides (processed from 10% buffered seawater formalin fixed tissues, embedded in paraffin using standard techniques, cut at 5µm thickness) from the Animal Health Laboratory, Mt Pleasant, DPIPWE, were reviewed by one pathologist, the author. To ensure reproducible results and uniform interpretation a subset of slides from each year was independently reviewed by two other pathologists. The agreement between descriptions and grades was calculated as a percentage of descriptions and grades, given by the second pathologist, which correlated with the descriptions and grades of the author.

Kidney (with the exception of 2003), heart, mantle, interstitium / leydig tissue, gonad, ganglia, gill, stomach, intestine, digestive gland and palp were examined. Histopathological findings were recorded and graded using a four point grading scale; 0=normal tissue with no microscopic changes evident, 1=minor alteration to organ architecture, < 1/3 of the organ, 2=changes affected and/or disrupted > 1/2 of the organ architecture, 3=severe changes with marked disruption or effacement of the majority of the organ architecture.

Statistical analysis

Chi–squared (χ^2) test of independence was used to determine if the relative frequency of oysters with histopathological changes differed a) across the years 2003 to 2005 due to the factor years and b) during the flood event of 2004 due to the factor mortality. The oysters sections examined from the flood event of 2004 were divided into 3 groups a)high mortality leases in Western Moulting Bay and Georges Bay (inter-tidal leases A-C) and the two low mortality leases b) Central Georges Bay (subtidal leases D & E) and c) Eastern Georges Bay (inter-tidal leases) (refer to Table 1). If the χ^2 analysis result was significant the standardised difference between the expected frequency and the observed frequency was used to interpret the frequencies.

Using a multivariate ANOVA test differences in the means of a) water quality variables in 2004 were analysed by the factors a) individual lease and b) lease group

(Table 1). The assumptions of homogeneity of variance were tested using Levene's Test. All tests were conducted at significance level P equal to or less than 0.05. All data were analysed using SPSS v18 (R).

RESULTS

Rainfall, George River flow and water salinity in Georges and Moulting Bay

In late January 226 mm of rain fell in just 3 days (from midnight 27th January 2004 to midnight 30th January) at St Helens aerodrome, on the eastern shore of Georges Bay. Upstream at Pyengana (the site of tributaries which flow into the George River) 197mm of rain fell for the 24 hours to 9am at 29th January 2004 (DPIWE 2004a). The estimated flow for the George River (which flows in to Georges Bay) was 675 cumecs (cubic metres per second) on 29th January 2004 and 30th January 2004. Comparing monthly average rainfalls in January and February across years, the late January rainfall in 2004 was significantly higher than that for most years from 2001 to 2008 (Figure 4a).

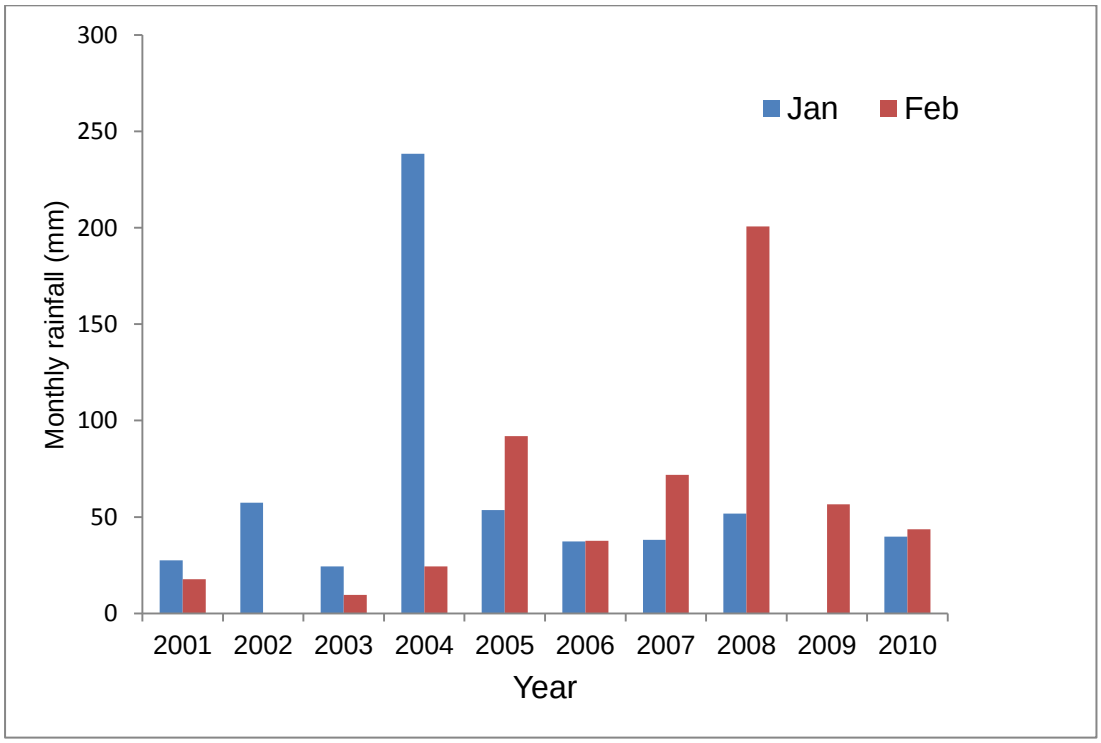

Figure 4a: Monthly rainfall for January and February from 2001 to 2010 (from Bureau of Meterology, Australia)

There was a high monthly rainfall in February 2008 but this was due to multiple rainfall events distributed through the month, including 45mm on 5th February, 29mm on 6th February and 70mm on 27th February. Comparing monthly rainfalls across the whole year from 2003 to 2005 the monthly rainfall in January 2004 was the highest over this period. There was a high monthly rainfall peak (208mm) in October 2005 (during the oyster sampling period) (Figure 4b) but this was spread over 2 weeks including peaks of 40mm from 9-10th October and 129.8mm, over 21st to 22nd October 2005.

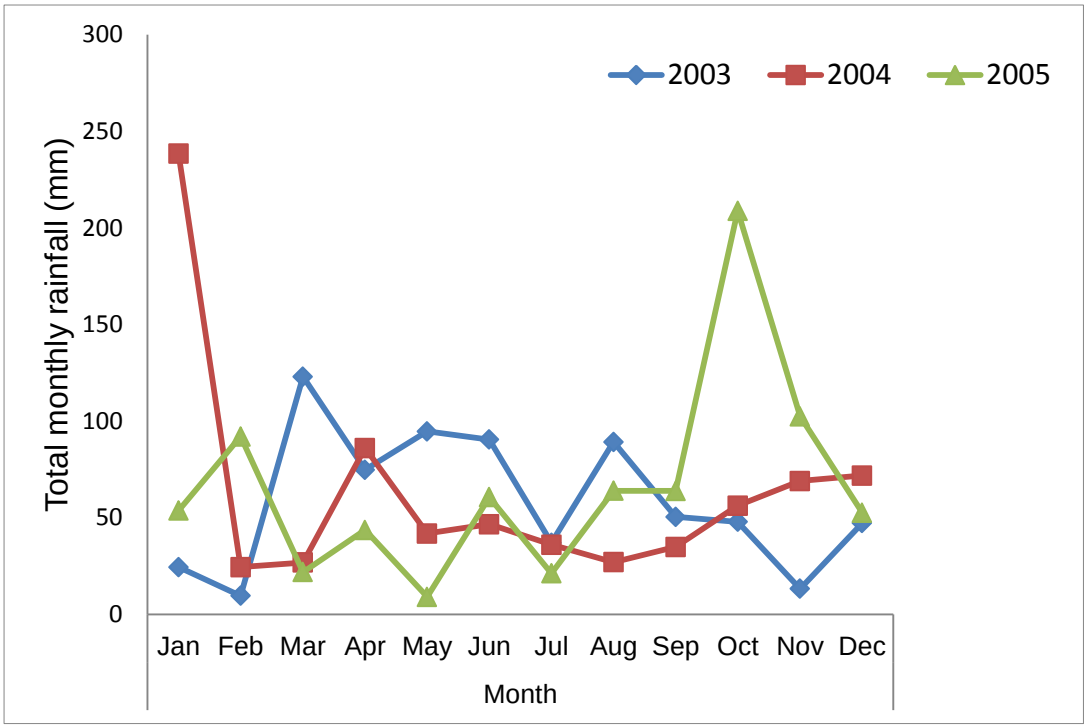

Figure 4b: Monthly rainfall (mm) for the years 2003 to 2005 at St Helens aerodrome (Bureau of Meteorology, Australia)

In late January and early February 2004 the substantial rainfall and greater than normal river flow resulted in freshwater flooding downstream into Georges Bay and Moulting Bay. On 30th January 2004 the automated TSQAP data logger from zone 6A/B (on leases F and G in eastern Georges Bay near the bay mouth) showed an abrupt decline in surface water salinity to 2ppt (Figure 5).

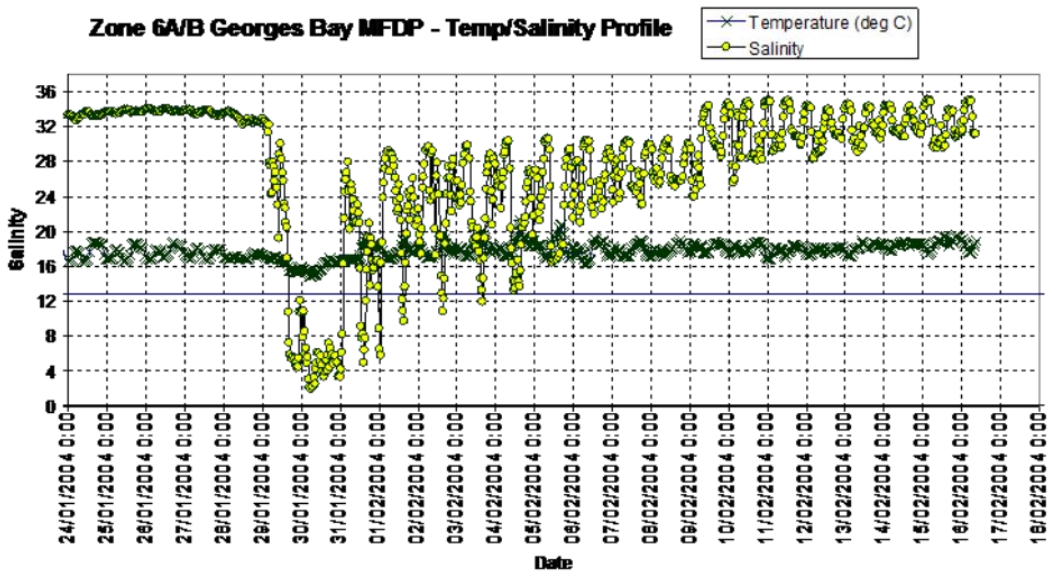

Figure 5: Salinity (ppt) and temperature (^{o}C) (the vertical axis has units for both temperature and salinity) measured by TSQAP data logger at Zone 6A/B (Leases F and G) (From DPIWE 2004a)

Tidal influences, post the flood event, elevated salinity to approximately 28 to 30ppt, but salinity quickly declined on outgoing tides. Depressed daily salinity conditions were recorded until 8th February, when minimum salinity was 28ppt and minor oscillations in values persisted at zone 6A/B until 16th February (DPIWE Tasmania 2004). There were no continuous salinity data available for the periods February 2003 and October to November 2005 but fortnightly salinity did not fall markedly and 7 day cumulative rainfall did not show any sudden high rainfall even similar to late January 2004 (Table 3).

Number of sites (n) sampled on each date	Water salinity (ppt) (mean ± SD)	Water temperature (^{o}C) (mean ± SD)	MF Thermotolerant coliform per 100ml across leases (mean ± SD)	MF Thermotolerant coliform per 100ml at sewage outflow	Rainfall past 7 days (mm)	Tide at time of sampling
2/02/2003 (n=15)	34.8 ± 0.5	20.1 ± 1.0	3 ± 0	3	30.8	mid falling
11/02/2003 (n=15)	34.7 ± 0.7	19.5 ± 0.5	3.5 ± 1.8	19	1.8	low falling
30/01/2004 (zone 6A, n=1)	2	16	not recorded	not recorded	226	low falling
17/02/2004 (n=15)	32.2 ± 1.4	20.2 ± 0.5	0.9 ± 0.04	1	6.6	low falling
12/10/2005 (n=15)	30.3 ± 2.2	14.4 ± 0.5	1.2 ± 0.9	4	53.8	low rising
24/10/2005 (n=8)	19.3 ± 3(shallow water) 24.2± 4.4(bay floor)	15.8 ± 0.7	not recorded	not recorded	129.8	not recorded
3/11/2005 (n=15)	25.0 ± 4.0	18.0 ± 1.0	4.7 ± 2.8	5	26	mid falling

Table 4: Water quality data, including water salinity, temperature and thermotolerant coliform counts across the 3 periods, 2003 to 2005, of sampling (Source of data TSQAP)

Pacific oyster mortalities and other history

During the freshwater flood event of 2004, significant mortalities of Pacific oysters occurred at the inter-tidal leases A-C in western Moulting Bay, near the mouth of the George river and oysters which were originally on inter-tidal lease C when the freshwater flooding began but were moved to sub-tidal lease D (in central Georges Bay) on 5th February. Histopathology samples were not taken of the oysters transferred from lease C to D. There were negligible mortalities amongst oysters set stocked (resident) on sub-tidal lease D, lease E, the other lease in central Georges

Bay, and inter-tidal leases F and G in eastern Georges Bay closest to the Barway at the mouth of the bay closest to the sea. There were no mortalities in 2003 on sub-tidal leases C and D, sampled as part of the annual TPOHSP. These stock (mean oyster size 55mm ± 5, SD) were transferred from Northwest Tasmania 2-3 months prior to sampling. There were no mortalities on leases A-G during the sampling program from October to November 2005 (mean oyster size 90mm ± 1, SD). Cumulative mortalities for each lease are shown in Table 5.

Lease	Location		Mean mortality ± SD (%)	Lease type	Oyster size (mm)
A	Western Moulting Bay and Georges Bay	High mortality	57 ± 18	inter-tidal	30-90
B			84 ± 31	inter-tidal	30-50
C			69 ± 26	inter-tidal	50-70
D*	Central Georges Bay	High mortality	87 ± 6	sub-tidal	50-60
D^		Low mortality	0.25 ± 0.25	sub-tidal	NR
E			0	sub-tidal	NR
F	Eastern Georges Bay	Low mortality	0	inter-tidal	NR
G			0	inter-tidal	NR

Table 5: A summary of the cumulative mortalities (counted on 12th and 13th February 2004) during the flood event 2004 by lease (data from DPIWE Tasmania 2004); NR=not recorded

*Racks transferred from lease C on 5th February 2004 to lease D.

^The balance of racks from lease D.

The cumulative mortality rates ranged from 49 to 95% at inter-tidal leases A-C in western Moulting Bay. Oysters which had been on lease C up until 5th February and then transferred to sub-tidal lease D (eastern Georges Bay), had similar high mean cumulative mortality of 87% ± 6 (mean ± SD). The cumulative mortality of all other oysters held only on lease D (in central Georges Bay) was very low, 0.25% ± 0.25 (mean ± SD). Mortalities were not recorded on the other sub-tidal lease in central Georges Bay, lease E, and inter-tidal leases F and G in eastern Georges Bay.

Environmental monitoring

In February 2004 the mean aluminium ($F=0.005$, $df=3, 13$ $P=1.000$), manganese ($F=1.715$, $df=3, 13$ $P=0.214$) and iron ($F=0.866$, $df=3, 13$ $P=0.483$) concentrations were all elevated across all lease groups and the mouth of the George River, compared to normal freshwater and marine levels (ANZGFMWQ 2000) (Table 6).

	Location								Marine water		Freshwater	
Water parameter	High mortality intertidal leases A-C 5, 7 and 12th Feb 2004 (n=9), mean ±SD		Low mortality subtidal leases D, E 7th and 12th (n=3) mean ±SD		Low mortality inter tidal leases F,G 7th Feb 2004 (n=3) mean ±SD		Near George river mouth 7th & 12th Feb 2004 (n=2) mean ±SD		normal parameters	levels reported to cause toxic effects in aquatic species	normal parameters	levels reported to cause toxic effects in aquatic species
Water pH	7.4	±0.3	7.7	±0.2	7.5	±0.2	7.7	±0.4	8.2		6.5-8.0	
Water conductivity (uS/cm)	28833	±7833	39067	±4852	28300	±12644	34750	±11243			100-5000	
Water Total Dissolved Solids (mg/L)	20421	±6897	25150	±4455	18780	±8792	21700	±6647				
Water Alkalinity CO3 (mg CaCO3/L)	<1		<1		<1		<1					
Alkalinity HCO3 mg CaCO3/L	67	±20	86	±10	64	±23	79	±28				
Alkalinity Total	68	±19	86	±11	65	±24	80	±29			>20	
Water Chloride (mg/L)	11311	±3535	16000	±2646	11367	±5631	14000	±5657				
Sulphate mg/L	1300	±394	1867	±321	1300	±624	1650	±636				
Water Hardness (mg CaCO3/L)	3488	±1199	5243	±999	3717	±1066	4650	±1584				
Water Ammonia (mg-N/L)	0.248	±0.259	0.213	±0.145	0.148	±0.076	0.086	±0.001	<0.1		<0.3 (> 16 degrees C)	
Nitrate + Nitrite (mg-N/L)	0.057	±0.062	0.047	±0.030	0.066	±0.037	0.109	±0.103	<100		<50	
Nitrate + Nitrite (mg-N/L)	0.06	±0.062	0.049	±0.032	0.069	±0.038	0.111	±0.104				
Nitrite mg-N/L	0.003	±0.002	0.003	±0.001	0.003	±0.001	0.002					
Phosphorus, Dissolved Reactive mg-P/L	0.01	±0.01	0.01	±0.00	0.01	±0.01	0.01		<0.05		<0.1	
Al Dissolved (µg/L)	50	±15	51	±17	50	±26	52	±15	<10	>2440	<30	>2300 (pH > 6.5)
Al Total µg/L	308	±151	123	±48	164	±20	222	±140				
As Dissolved µg/L	<5		<5		<5		<5		<50	>893	<50	>961
As Total µg/L	<5		<5		<5		<5					
Cd Dissolved ug/L	<1		<1		<1		<1		<5		<5	
Cd Total µg/L	<1		<1		<1		<1					
Co Dissolved µg/L	<1		<1		<1		<1			>45		>2.8
Co Total µg/L	<1		<1		<1		<1					

1301-Water Cr Dissolved (ug/L)	<1		<1		<1		<1			> 10300		>430
Cr Total µg/L	<1		<1		<1		<1					
Cu Dissolved µg/L	<1		<1		<1		<1		<5	> 0.4 to 20000^	<5	> 1.64
Cu Total µg/L	<1		<1		<1		<1					
Fe Dissolved µg/L	30	±12	20	0	29	±16	20	0	<10		<10	> 300
Fe Total µg/L	364	±235	107	±56	148	±28	225	±195				
Mn Dissolved µg/L	27	±11	11	±8	20	±8	18	±18	<10		<10	
Mn Total µg/L	37	±19	21	±11	43	±26	35	±18				
Ni Dissolved µg/L	<2		<2		<2		<2		<100		<100	
Ni Total µg/L	<2		<2		<2		<2					
Pb Dissolved µg/L	<10		<10		<10		<10		<10		<10	
Pb Total µg/L	<10		<10		<10		<10			>25		>28
Zn Dissolved µg/L	340	±1098	4	±3	4	±3	4	±3	<6	> 400-1760	> 5 to 15	
Zn Total µg/L	5	±4	5	±3	5	±2	4	±2				
1302-Water Ca Dissolved (mg/L)	210	±75	322	±65	223	±±66	284	±103				
Ca Total mg/L	248	±79	368	±78	275	±75	320	±108				
K Total mg/L	222	±75	340	±75	249	±70	283	±118				
Mg Dissolved mg/L	720	±246	1078	±204	767	±219	955	±319	<15		<15	
Mg Total mg/L	748	±253	1133	±2±51	826	±248	973	±350				
Na Total mg/L	6306	±2046	9240	±1565	7047	±1822	7830	±2659				

Table 6: Water quality data by lease including heavy metals, Water quality data for 5th to 12th February (source of data DPIWE 2004a; normal parameters and toxicity levels were from ANZGFMWQ 2000)

The mean rises in aluminium, manganese and iron were not at levels reported to cause toxicity in aquatic organisms (ANZGFMWQ2000). Normal dissolved aluminium levels for both marine and freshwater for continuous exposure range from <10µg/L (Meade, 1989 cited in ANZGFMWQ 2000) to <30µg/L at pH levels of greater than 6.5 (ANZGFMWQ 2000).

The concentration of dissolved zinc on one lease, lease C (western Georges Bay near the mouth of the George River) on 5th February was 3560 µg/L. This was significantly higher than any other site sampled. The means for the other intertidal leases near the river mouth (leases A and B), intertidal and sub tidal leases and the mouth of the George River were all 4±3 µg/L (mean ± SD). By 7th February dissolved zinc levels reduced to 50µg/L. All this time there was no elevation in dissolved zinc other leases. By 12th February dissolved zinc on lease C was 3µg/L.

Pesticides (including tributyl tin) and heavy metal levels in the water during the flood event of 2004 were below recommended levels (ANZGFMWQ 2000) (Appendix Table A1 and A2). Heavy metal concentrations in oyster meat from 2003 and 2005 were not above recommended levels (personal communications TSQAP) (Appendix Table A3).

Algal species, representing the natural flora for Georges Bay and at lower than normal micro algal cell abundance, was identified from water samples collected across multiple leases on 5th to 12th February 2004 are listed in the Appendix in Table A4 (DPIWE Tasmania 2004). The only potentially harmful species present included *Prorocentrum rathymumn*, which is normally present in far greater abundances than at the time of sampling" (DPIWE Tasmania 2004). Algal results for 2005 were also unremarkable, (A. Turnbull pers. comm.) (Appendix Table A5). For February 2003 algal samples were not collected for Georges and Moulting Bay.

Gross findings and Microbiology

Gaping shells were found on leases A –D (refer to mortality rates recorded Table 4). Eight oysters sampled from lease E had raised 1-2 cm diameter raised fluid filled blisters on the inner surface of the shell lined by fragile nacre. Another oyster from

lease E had a shell blister, a 1cm diameter yellow fluid filled raised defect on the inner shell lined by fragile nacre. In 2003 and 2005 there were no abnormal shells or significant defects, which affected the integrity of the shell and the shell seal or gross lesions such as changes in colour, shape or texture of the oyster meat.

During the 2004 flood event no significant aquatic bacterial pathogens were isolated. From lease E mixed *Vibrio* spp. were isolated from shell abscess in one oyster. Four oyster meat samples from lease C grew mixed bacteria including *Clostridium* spp, *Bacteroides* spp and *Porphyromonas* spp. There was no bacterial growth from 6 oyster haemolymph sampled from each lease E, F and G and 10 haemolymph samples from lease C. Microbiology samples were not taken for oysters from 2003 or 2005 (DPIWE Tasmania 2004).

Histopathology

There were microscopic changes in the stomach and intestines (χ^2=99.347, df 2; P<0.001), digestive gland (χ^2=213.856, df 2; P<0.001), interstitium and leydig tissue (χ^2=225.414, df 2; P<0.001), mantle (χ^2=183.129, df 2; P<0.001), kidney (χ^2=29.254, df 2; P<0.001), gonads (χ^2=30.422, df 2; P<0.001) in oysters sampled during the February 2004 freshwater flood and these were significantly more common in February 2004 than 2003 or 2005 (Table 7 & Table A6). In the stomach and intestines there were diffuse mild intramural infiltrating haemocytes (diapedesis) and expanded intercellular spaces (interstitial oedema) distending the gastric (Figure 6b and 6c) and intestinal walls and many epithelial cells contained intracytoplasmic vacuoles (grade 1-2). In the digestive glands there were expanded intercellular spaces (grade 1) and dilated digestive gland tubules (grade 1) (Figure 6e). Intercellular spaces and haemolymph vessels in interstitial tissues were expanded (grade 1) in the mantle and leydig tissue. There was leydig cell necrosis with occasional haemocyte infiltrate (grade 1-2) and multifocal mantle erosion (grade 1-2) (Figure 6g). Within the gonad of female oysters intercellular spaces were expanded and there was haemocyte infiltrate (often associated with post spawning) (grade 1). Many oysters had spawned and there was necrosis of ova in the gonads of some oysters. In the kidney there were expanded intercellular spaces, intracytoplasmic vacuoles in epithelial cells or dilated renal tubules (grade 1) (Figure 6i). As the

frequency of grades 1 and 2 for histopathological changes did not differ between treatment groups based on year, lease group or individual lease ($P > 0.05$), grades 1 and 2 were pooled for each histopathological description (Tables 7 and 8, Appendix Tables A6 and A7).

Histopathological findings	2003 (n=0)	2004 (n=178)	2005 (n=288)
Kidney			
Expanded intercellular spaces, intracytoplasmic vacuolation of epithelia cells or dilated tubules	NA	49↑	25↓
Histopathological findings	2003 (n=63)	2004 (n=178)	2005 (n=288)
Stomach and intestines			
Expanded intercellular spaces in stomach and intestines walls and intracytoplasmic vacuoles and haemocyte infiltrate into wall	0↓	46↑	0↓
Digestive glands			
Atrophied digestive gland tubules lined by low cuboidal cells, expanded extracellular spaces / tubular necrosis	0↓	90↑	0↓
Gonad			
Expanded intercellular spaces and haemocyte infiltrate of gonad / ova necrosis	0↓	15↑	0↓
Post spawn	0↓	47↑	0↓
Mantle			
Multifocal erosion of the mantle	0↓	96↑	0↓
Interstitium			
Expanded intercellular spaces of the interstitium / necrosis of leydig cells with occasional haemocyte infiltrate	0↓	94↑	2↓

Table 7: The numbers of Pacific oysters from each year, with significant histopathological findings; Arrows indicate if observed values are above or below expected values. The numbers listed below are total numbers for each year and combine grades 1 to 2 for histopathological changes. NA = not applicable; there were no kidney sections available in 2003.

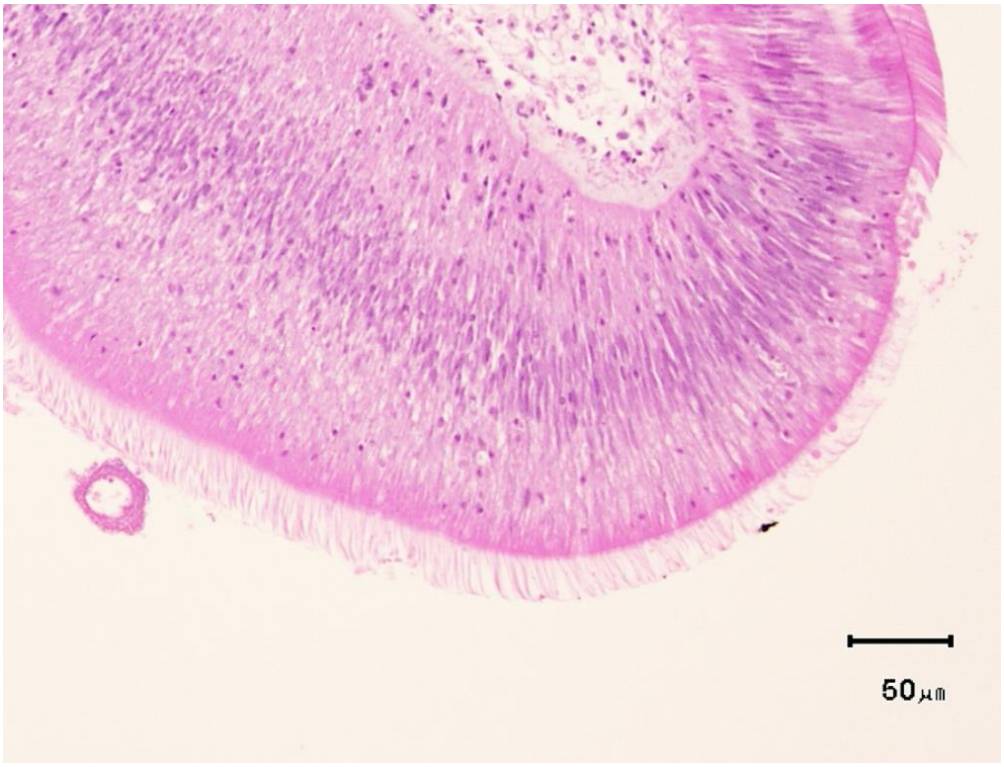

Figure 6a: Normal stomach from lease A in 2005

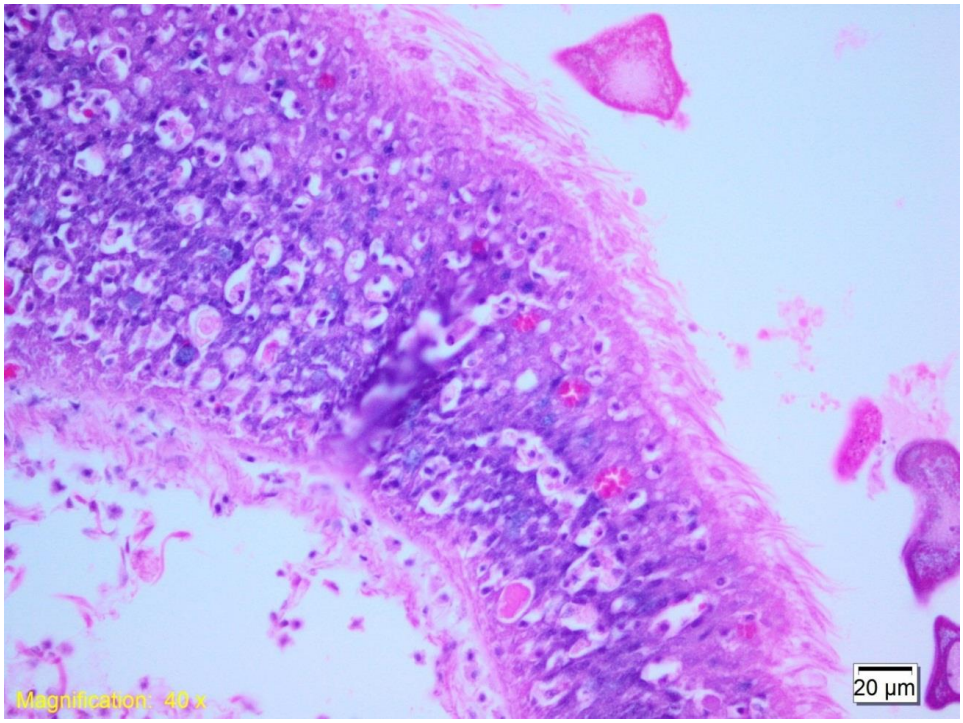

Figure 6b: Expanded intercellular spaces with haemocyte infiltrate in the stomach, grade 1 (lease A, 2004)

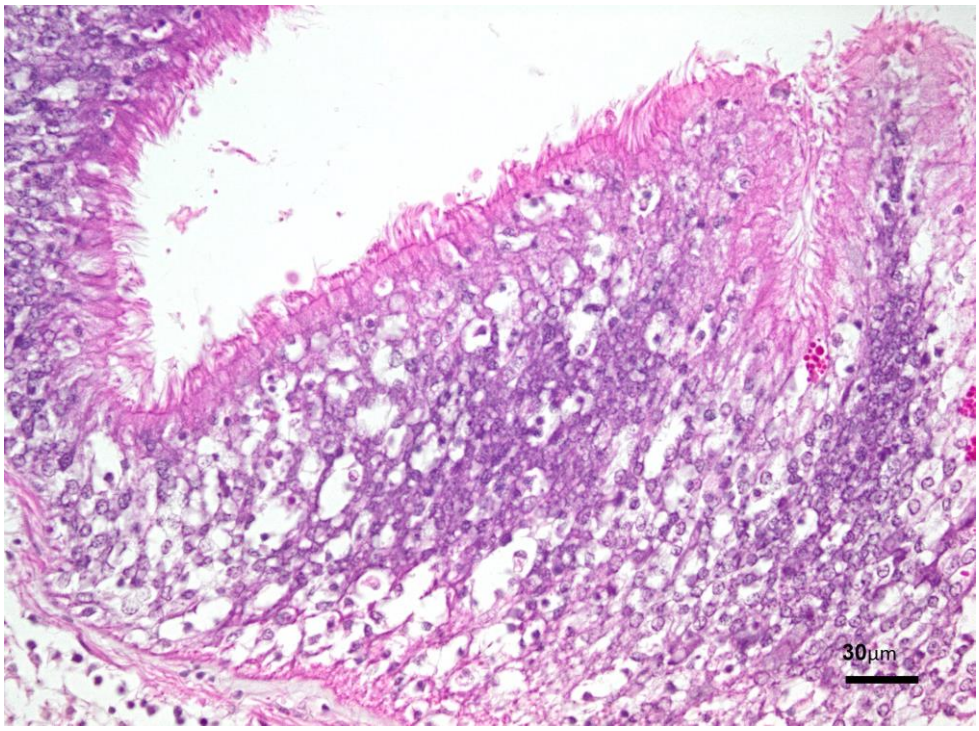

Figure 6c Expanded intercellular spaces with haemocyte infiltrate in the stomach, grade 2 (lease A, 2004)

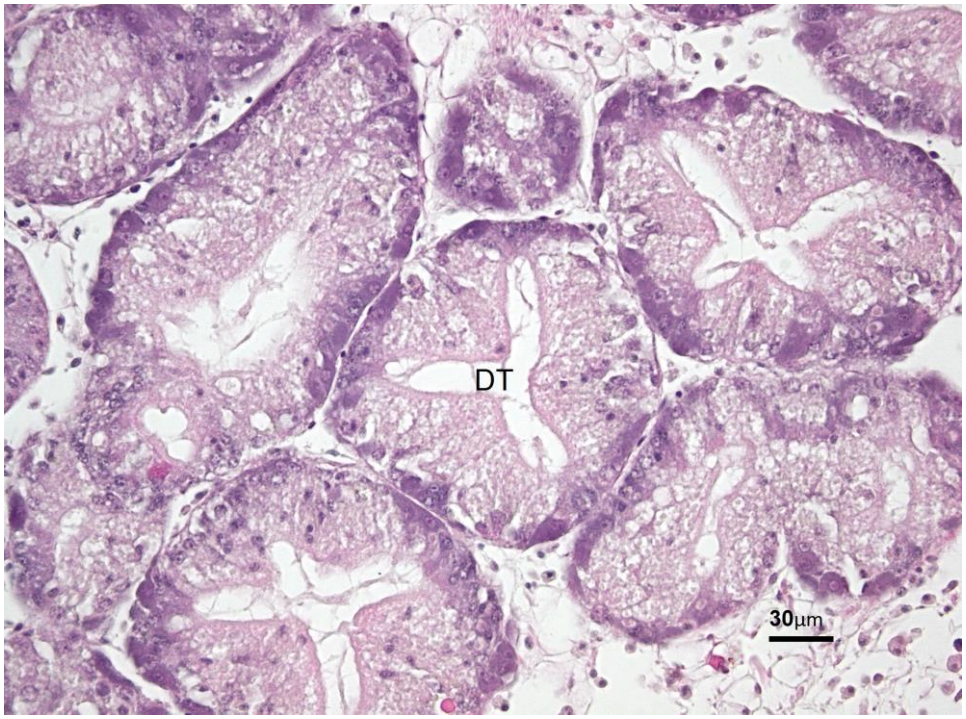

Figure 6d: Digestive gland with normal digestive tubules (DT), from lease D 2004

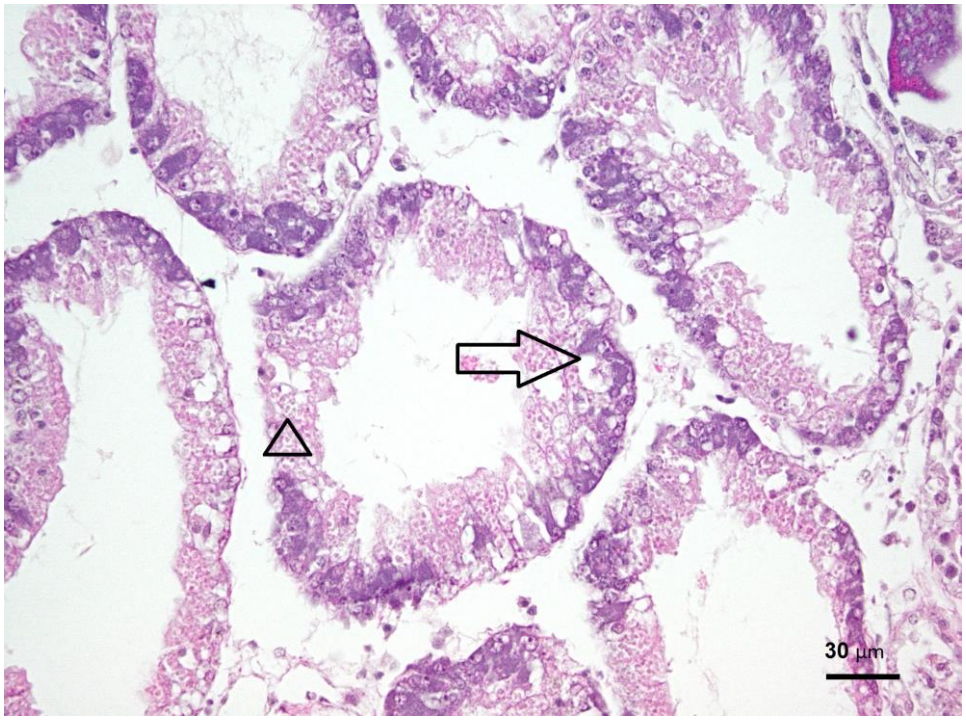

Figure 6e: Atrophied digestive tubules with expanded intercellular spaces (arrow head) and intracytoplasmic vacuoles (arrow), grade 1, lease A 2004

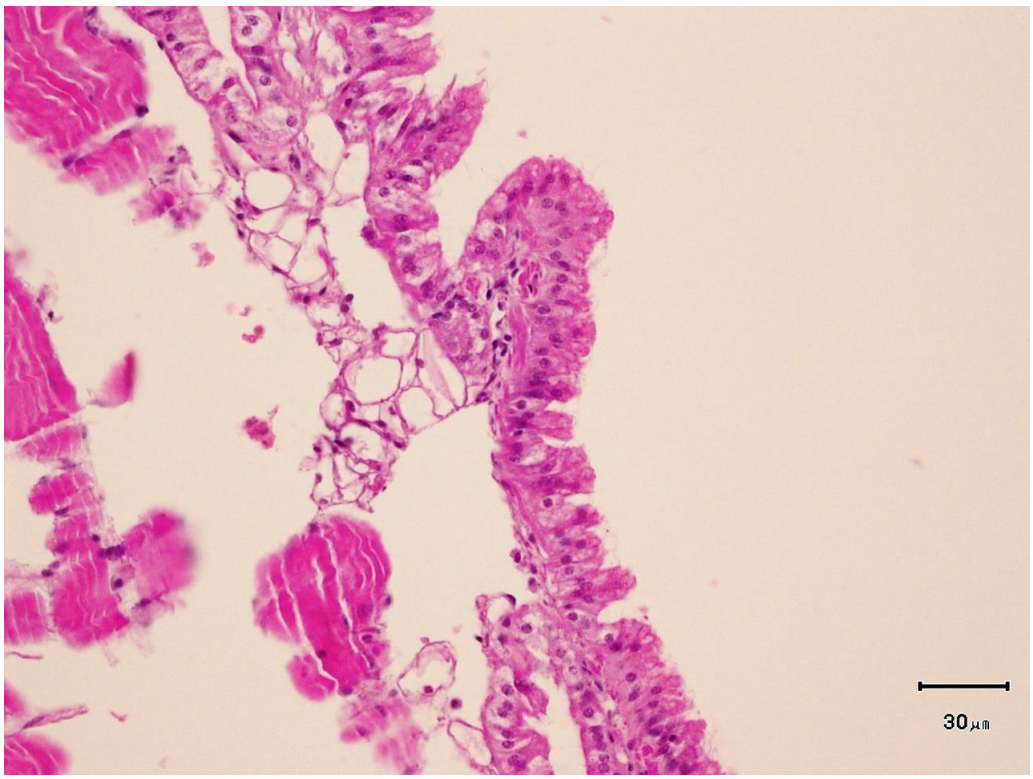

Figure 6f: Normal mantle epithelium, lease C 2003,

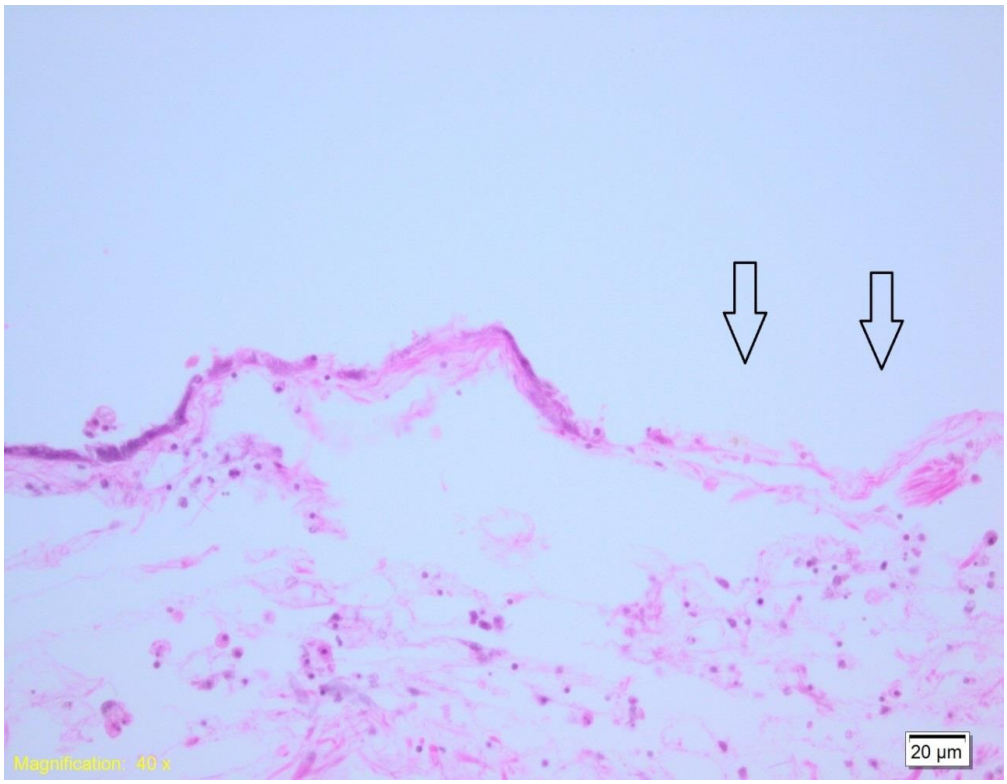

Figure 6g: Mantle erosion with loss of epithelium, grade I (lease B, 2004)

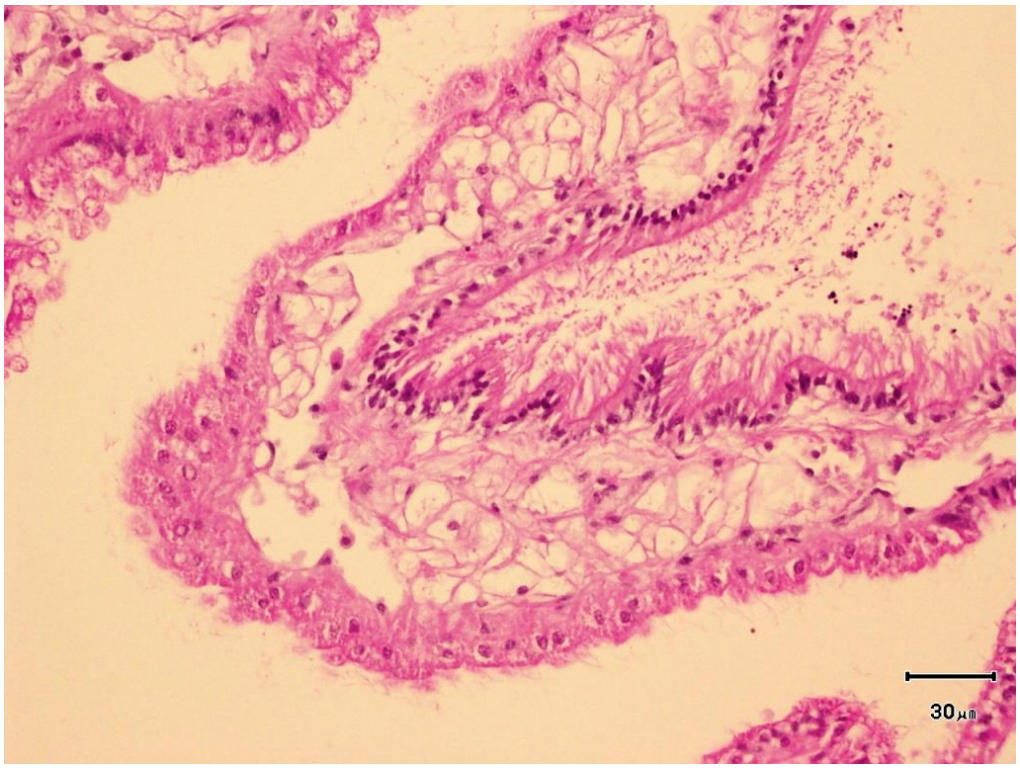

Figure 6h: Normal kidney from lease C 2005

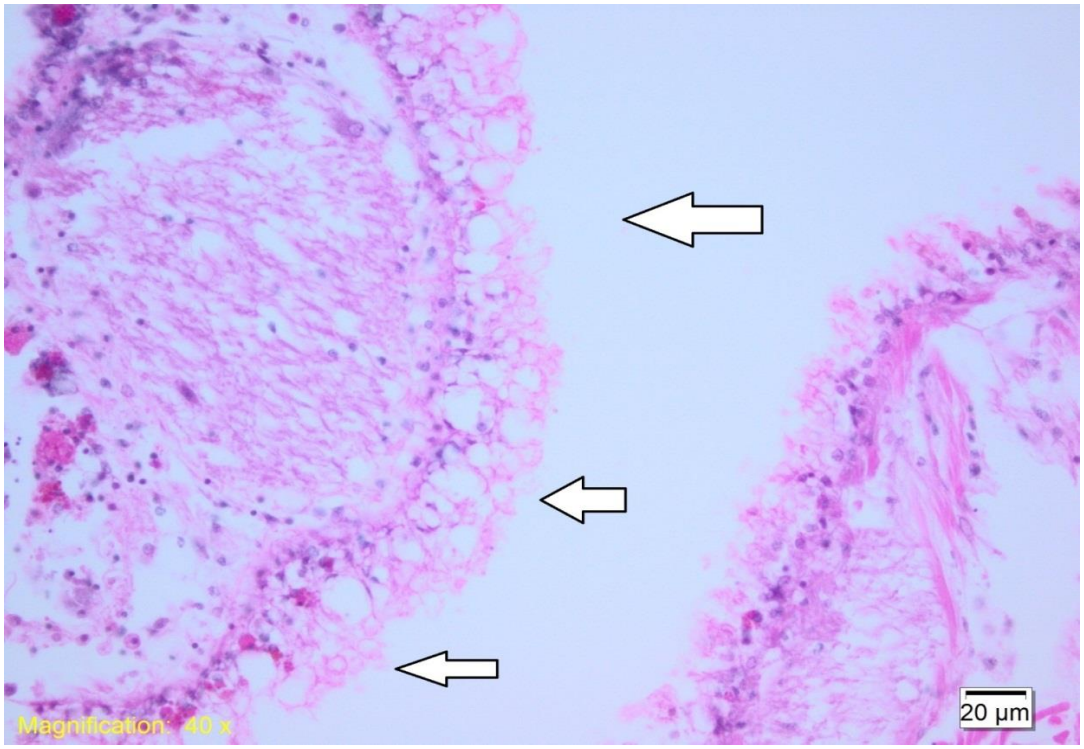

Figure 6i: Expanded intercellular spaces (arrows) in kidney, grade 1 (lease A, 2004)

During the 2004 flood event significantly more oysters sampled from high mortality western leases A-C showed expanded intercellular spaces, intracytoplasmic vacuoles and dilated tubules in the kidneys (χ^2=25.333, df 2; P=0.001) and multifocal mantle erosions (χ^2=17.707, df 2; P<0.001) than oysters across the other leases with low mortalities (Table 8). Leydig cell necrosis was seen across all leases but most frequently in sub-tidal leases in eastern Georges Bays. Sub-tidal oysters in eastern Georges Bay (leases D and E) had more frequently interstitial oedema (χ^2 =12.957, df 2, P=0.002), oedema and haemocyte infiltrate in the gonad (χ^2 =52.533, df 2, P< 0.0001) and expanded intercellular spaces with haemocyte infiltrate in the stomach and intestines (χ^2 =96.983, df 2, P<0.0001). Inter-tidal oysters from Georges Bay (leases D and E) had more frequent digestive gland atrophy with expanded intercellular spaces, intracytoplasmic vacuoles and haemocyte infiltrate (χ^2 =12.372, df 2, P=0.002). Within groups of leases, there was no significant difference in the frequency of histopathological findings between individual leases in February 2004 (P> 0.05).

Histopathological findings	Intertidal Western Moulting /Georges Bay (Leases A-C) (n=109)	Intertidal Georges Bay (Leases D & E) (n=37)	Sub tidal Eastern Georges Bay (Leases F & G) (n=32)
Kidney			
Expanded intercellular spaces, intracytoplasmic vacuolation of epithelia cells or dilated tubules	44↑	0	5↓
Stomach and intestines			
Expanded intercellular spaces in stomach and intestines walls and increased numbers of intracytoplasmic vacuoles and haemocyte infiltrate into wall	16↓	0↓	30 ↑
Digestive glands			
Atrophied digestive gland tubules lined by low cuboidal cells, expanded intercellular spaces / tubular necrosis	53↓	27↑	10↓

Table 8: The numbers of Pacific oysters from lease groups with histopathological findings during 2004 flood event. Arrows indicate if observed values are above or below expected values. The numbers listed below are total numbers for each lease group and combine grades 1to 3 histopathological changes.

Histopathological findings	Intertidal Western Moulting /Georges Bay (Leases A-C) (n=109)	Intertidal Georges Bay (Leases D & E) (n=37)	Sub tidal Eastern Georges Bay (Leases F & G) (n=32)
Gonad			
Expanded intercellular spaces and haemocyte infiltrate of gonad / ova necrosis	1↓	0↓	13↑
Post spawn	0↓	47↑	0↓
Mantle			
Multifocal erosion of the mantle	70↑	9↓	17
Interstitium			
Expanded intercellular spaces of the interstitium / necrosis of leydig cells with occasional haemocyte infiltrate	56↓	13↓	25↑

Table 8 continued

For 100% of slides reviewed by both the author and second pathologist there was agreement in descriptions and grades.

There were no histopathological findings consistent with iridovirus, marteiliosis, bonamiosis, haplosporidiosis, perkinsiosis, nocardiosis and mikrocytosis in oysters from the retrospective study.

DISCUSSION

Mortalities of Pacific oyster, after freshwater flooding, can be the result of multiple pluviometric (water borne) factors (Ropert et al. 2008). These factors include sudden fall in salinity, increased suspended sediment, decreased dissolved oxygen, pesticides, heavy metals, eutrophication and acid sulphate soil run off (Dove 2003; Ropert et al. 2008). Often a combination of two or more of these stressors

overwhelms oysters during freshwater flooding and lead to mortality events (Ropert et al 2008). Finally additional inter-current seasonal stressors, such as warmer water temperature in summer, may be the final factors that determines if the oysters survive freshwater flooding or die (Shumway 1996).

A key stress factor for oysters during the February 2004 flood event in Georges and Moulting Bay was the abrupt fall in salinity. The magnitude of the flood event and sudden fall in salinity was uncommon for this region which usually had stable salinity (DPIWE Tasmania 2004). During a year-long study, April 1993 to February 1994 the variation in salinity across sites in Georges and Moulting bays was limited to 3ppt. The lowest salinity record (31.5ppt) generally occurred near the George river mouth. The sites near the eastern mouth of the Georges Bay had very little variation in salinity, persistently 34-35ppt (Crawford and Mitchell 1999).

In oysters sampled during the February 2004 floods there were histopathological changes such as leydig tissue breakdown and necrosis suggesting metabolic stress (Galtsoff 1964). Oysters and other marine bivalve molluscs withstand sudden falls in salinity by closing their shell valves to protect themselves from sudden exposure to extreme falls in salinity (Hoyaux et al. 1976; Hand and Stickle 1977; Davenport 1981). These processes are initiated by the sudden change in intracellular sodium concentration (Natochin et al. 1979) because oysters have an open circulation system (Galtsoff 1964). Because leydig tissue contains abundant stored glycogen (Grizel 2003) leydig tissue necrosis in oysters during the flood event most likely indicated limited filter feeding and increased catabolism of metabolic energy stores (Galtsoff 1964) due to their shells being closed. Leydig cell necrosis was more common in subtidal low mortality leases in eastern Georges Bay suggesting these oysters remained closed longer than oysters on high mortality leases.

In February 2004, many oysters had atrophied digestive gland tubules lined by low cuboidal cells and dilated lumens (particularly on subtidal leases D and E), which were also consistent with closed shells and fasting, during the initial insult of freshwater (Winstead 1995). Similar digestive gland atrophy was described in Eastern oysters (*C. virginica*) following prolonged (3 week) freshwater flooding in

Apalachicola Bay, Florida (Winstead 1995). Although the oysters were sampled just 7-9 days post flooding, in Georges and Moulting Bay, the environmental stressor was the same and fasting for as short as 48 hours will produce digestive gland atrophy (Winstead 1995). Similarly, digestive gland atrophy was associated with fasting or lack of feeding caused by seasonal decrease in phytoplankton, measured by chlorophyll a concentration in sea water, in Pacific oysters in Gamakman Bay of the South Korean coast (Kang et al. 2010), and regional shifts in temperature (extrapolated to decreased phytoplankton numbers and fasting) associated with population declines in surf clams (*Spisula solidissima*) off the Delmarva Peninsula, near Delaware, USA (Kim and Powell 2004). Chlorophyll a was not measured in the waters around the leases during the mortality investigation at Georges and Moulting Bay in 2004. Other environmental factors which cause digestive gland atrophy in marine bivalves but can be excluded from the Georges and Moulting bay mortalities, based on water testing, include exposure to *P. rhathymum* (a harmful algae) in Pacific oysters (*C. gigas*) (Pearce et al. 2005) (*P. rhathymum* was not detected in elevated numbers or concentrations during the Georges and Moulting Bay floods of 2004), oil spills in mussels (*M. edulis*) (Neff and Haensley 1982; Widdows et al. 1982) (no oil spills were reported associated with the oyster mortalities at Georges Bay) or suspected pollutants in water discharged from iron and steel factories, also in mussels (*M. edulis*)) (Sunila 1987) (there are no iron or steel factories in the Georges and Moulting Bay areas or along the George river).

In some oysters during the February 2004 flood there was digestive gland necrosis but a cause for this was not identified based on water data results. Digestive gland necrosis in *Mytilus edulis* has been associated with heated water effluent from power stations (Gonzalez and Yevich 1976) or high levels of cadmium (Gold-Bouchot et al. 1995) but there were no power plants in the area and no rise in cadmium on water testing in Georges and Moulting Bays.

Overtime oysters and other marine bivalve molluscs (such as *Mytilus edulis, Scrobicularia plana, Glycymeris glycymeris*) will eventually open in low salinity due to metabolic and respiratory demands and will osmoconform (Hoyaux et al. 1976; Hand and Stickle 1977; Davenport 1981). In high mortality leases (A-C) oysters had

expanded intercellular spaces between renal epithelium more often than oysters from low mortality leases (D-G). In *Mytilus* sp. challenged with sudden low salinity water similar expanded intercellular spaces in the kidney occurred (Khan and Saleuddin 1986) as the mussels osmoconformed. This suggests the renal changes in oysters from leases A-C were associated with osmoconformation. During the February 2004 flood event there were similar osmoconforming changes in the alimentary tract and interstitium. There were expanded intercellular spaces in the stomach, intestine, digestive gland and interstitium across all leases.

In oysters from the February 2004 floods intracytoplasmic vacuolations in epithelial cells and mural haemocyte infiltrate (diapedesis) in the intestines and stomach were more abundant and prominent than oysters from 2003 and 2005. Intracytoplasmic lysosomal vacuoles are part of the normal digestion process for bivalve molluscs (Florey 1966). Low salinity water elevates metabolic demand in oysters such as *C. virginica* (Shumway 1996). Taking this into account, increased numbers or more prominent intracytoplasmic vacuoles in oysters extracted from flooded Georges and Moulting Bays in 2004, suggested increased metabolic demand and activity.

The dispersion of freshwater across Georges Bay and Moulting Bay is dependent on wind direction and tidal stage (Brown 1998). If these off shore winds and tides resulted in uneven distribution of the freshwater stress factors through the estuary, for example, persistent lower salinity (or other freshwater associated stress factors) near the river mouth close to intertidal leases A-C, this may partially explain the higher mortalities across inter-tidal leases A-C in western Moulting Bay (see figure 2). Unfortunately, extensive measurements comparing salinity and other factors such as suspended sediment across the various leases during the flood event were not available to support this hypothesis.

There is insufficient environmental data to explain why not all microscopic changes were most commonly associated with mortality in 2004. Other concurrent environmental stress factors, associated with flooding, such as low dissolved oxygen or temperature variation at different depths, which were not measured during the investigation, may have contributed to some changes being more common in sub-

tidal compared to inter-tidal leases or low mortality compared to high mortality leases. For example, osmoconforming changes in the alimentary tract were common in low mortality compared to high mortality leases possibly because of variations in dissolved oxygen (influenced by floodwaters, and tidal patterns) across different leases in the bays. On the other hand, breakdown and necrosis of leydig tissue may have been more common in sub-tidal leases than inter-tidal leases because water temperature and salinity, post flooding, varied with depth. The sub-tidal oysters were kept deeper in the water (up to 6 to 10 metres below the water's surface at high tide).

In February 2004, in addition to the abrupt fall in salinity there was seasonal elevation in water temperature. During summer, oysters, which are poikilotherms, have high metabolic and respiratory rates (Galtsoff 1964). For this reason the metabolic and respiratory rates of oysters would have been high, during the February 2004. As mentioned before, exposure to low salinity also elevates metabolic demand (Shumway 1996). This combined effect of low salinity and increased temperature on metabolic rate may have forced some oysters, particularly those which were inter-tidal (and exposed to higher temperatures and lower salinities – because they were higher in the water - than sub-tidal oysters), to open their shells in the low salinity water to feed and obtain oxygen.

An additional potential freshwater stress factor identified during the February 2004 floods was zinc. Zinc was markedly increased (dissolved zinc 3560 µg /L) on lease C on 5th February 2004. However there was a rapid reduction in dissolved zinc levels to 50 micrograms/L on 7th February. Pacific oysters (*C. gigas*) are sensitive to high levels of zinc (10000 µg /L) over short periods of time (7 days) and will die but they accommodate to moderate levels (5000 µg /L) of zinc (Mottin et al. 2012). Based on these published results the short term peak on lease C, which was below 10000ug/L probably didn't cause the mortalities alone. Zinc can enter surface waters as a result of run off from mining enterprises (ANZGFMWQ 2000), however, no active mines are found around Georges or Moulting Bays or George River. Another source of zinc is acid sulphate soil run-off from agricultural land. Acid sulphate soils are found in the George River catchment (DPIPWE 2010).

Acid sulphate soils are present in coastal lands near the mouth of the George River (DPIPWE 2010) and run off from acid sulphate soils was potentially a risk factor for oyster mortalities during the freshwater flood in February 2004. Elevated iron, zinc and aluminium in the flood waters, which can be associated with sulphuric acid release from the soil following heavy rains (Dove 2003; DPIPWE 2010), were recorded during the flood (DPIWE 2004a). However, characteristic microscopic changes in oysters in response to acid sulphate soil run off, such as inflammation of the gills and mantle (Dove 2003), were not found in oysters from Georges Bay in February 2004. In summary, there were some water quality changes, including increases in aluminium, zinc and irons, suggestive of acid sulphate soil runoff but no microscopic changes in the oysters to confirm the mortalities were related to acid sulphate soil run-off.

Acute mantle erosion was another significant microscopic finding and was seen more commonly in oysters on high mortality inter-tidal leases A-C, than on other leases. The mantle primarily acts as a barrier against chemical and physical injury, secretes shell and ligament and along with other organs mediates the conversion from aerobic to anaerobic metabolism (Grizel 2003). For these reasons the acute mantle erosion in oysters from the February 2004 flood event not only caused a break down in the physical barrier to freshwater but also may have compromised oysters' ability to adapt to changes in metabolism and feeding when they opened again. Mortalities (up to 90%) of pearl oysters (*Pinctada maxima)* were reported in October 2006 in Exmouth Gulf, Western Australia and mantle erosion was a common finding in the majority of the moribund oysters but unfortunately the cause for the mortalities was not identified (Jones et al. 2010). A possible cause for mantle erosion in Pacific oysters (*C. gigas*) from the February 2004 floods, which was not identified by environmental monitoring, was mechanical damage due to fine suspended sediment, washed down by the flooded George River.

When assessing microscopic changes in oysters it is important to exclude, as far as practical, the contribution of infectious pathogens. In the oysters from 2003 to 2005 there were no histopathological findings consistent with infectious diseases such as Iridovirus (Elston and Wilkinson 1985), Marteiliosis (Thebault et al. 2005)

Haplosporidiosis (Perkins 1968), Perkinsiosis (Villalba et al. 2004), Nocardiosis (Friedman 1990), Mikrocytosis (Hervio et al. 1996) and Bonamiosis (Hine et al. 2001) which is endemic in Tasmanian flat oysters (*O. angasi*) in the oysters from Georges and Moulting Bays. At the time of the mortality event in February 2004 samples were not tested for Ostreid Herpesvirus 1 (OsHV1) by PCR. Subsequently, in 2011 a state-wide survey of all Pacific oyster growing areas in Tasmania detected no evidence of OsHV1 by PCR (Ellard 2011). Additional state-wide surveys in 2012 and 2013 also have not detected OsHV1 by PCR in Pacific oysters (Ellard 2012; Ellard 2013).

Conclusion

The purpose of this retrospective study was only to assess the histopathological changes in light of water quality data. This was a limited investigation and could not identify all the multiple causes leading to the oyster mortality for many reasons. For example, sudden physiological stressors, which cause very little histopathological changes, such as sudden falls to low dissolved oxygen, could not be interpreted by histopathology. In addition dead, autolysed oysters, which were obviously susceptible to the freshwater associated lethal stressors, were unsuitable for histopathology and thus unable to be assessed.

In summary, based on the retrospective review of histopathology slides, associated with the stress of abrupt change to low salinity and summer temperatures, there were osmoconforming (expanded intercellular spaces in the kidney and alimentary tract) and metabolic stress (leydig tissue necrosis) related changes in Pacific oysters, during the February 2004 floods.

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APPENDIX

	Location							
Water parameter	High mortality intertidal leases 5, 7 and 12th Feb 2004 (n=9)		Low mortality intertidal leases 7th and 12th (n=3)		Low mortality subtidal leases 7th Feb 2004 (n=3)		Near George river mouth 7th & 12th Feb 2004 (n=2)	
Water pH	7.4	±0.3	7.7	±0.2	7.5	±0.2	7.7	±0.4
Water conductivity	28833	±7833	39067	±4852	28300	±12644	34750	±11243
Water Total Dissolved Solids (mg/L)	20421	±6897	25150	±4455	18780	±8792	21700	±6647
Water Alkalinity CO3 (mg CaCO3/L)	<1		<1		<1		<1	
Alkalinity HCO3 mg CaCO3/L	67	±20	86	±10	64	±23	79	±28
Alkalinity Total	68	±19	86	±11	65	±24	80	±29
Water Chloride (mg/L)	11311	±3535	16000	±2646	11367	±5631	14000	±5657
Sulphate mg/L	1300	±394	1867	±321	1300	±624	1650	±636
1109-Water	3488	±1199	5243	±999	3717	±1066	4650	±1584
Water Ammonia (mg-N/L)	0.248	±0.259	0.213	±0.145	0.148	±0.076	0.086	±0.001
Nitrate + Nitrite (mg-N/L)	0.057	±0.062	0.047	±0.030	0.066	±0.037	0.109	±0.103
Nitrate + Nitrite (mg-N/L)	0.060	±0.062	0.049	±0.032	0.069	±0.038	0.111	±0.104
Nitrite mg-N/L	0.003	±0.002	0.003	±0.001	0.003	±0.001	0.002	
Phosphorus, Dissolved Reactive mg-P/L	0.01	±0.01	0.01	±0.00	0.01	±0.01	0.01	
Al Dissolved (µg/L)	50	±15	51	±17	50	±26	52	±15
Al Total ug/L	308	±151	123	±48	164	±20	222	±140
As Dissolved ug/L	<5		<5		<5		<5	
As Total ug/L	<5		<5		<5		<5	
Cd Dissolved ug/L	<1		<1		<1		<1	
Cd Total ug/L	<1		<1		<1		<1	
Co Dissolved ug/L	<1		<1		<1		<1	
Co Total ug/L	<1		<1		<1		<1	
1301-Water Cr Dissolved (ug/L)	<1		<1		<1		<1	
Cr Total ug/L	<1		<1		<1		<1	
Cu Dissolved ug/L	<1		<1		<1		<1	
Cu Total ug/L	<1		<1		<1		<1	
Fe Dissolved ug/L	30	±12	20	0	29	±16	20	0
Fe Total ug/L	364	±235	107	±56	148	±28	225	±195
Mn Dissolved ug/L	27	±11	11	±8	20	±8	18	±18
Mn Total ug/L	37	±19	21	±11	43	±26	35	±18
Ni Dissolved ug/L	<2		<2		<2		<2	
Ni Total ug/L	<2		<2		<2		<2	
Pb Dissolved ug/L	<10		<10		<10		<10	
Pb Total ug/L	<10		<10		<10		<10	
Zn Dissolved ug/L	4	±3	4	±3	4	±3	4	±3
Zn Total ug/L	5	±4	5	±3	5	±2	4	±2
1302-Water Ca Dissolved (mg/L)	210	±75	322	±65	223	±±66	284	±103
Ca Total mg/L	248	±79	368	±78	275	±75	320	±108
K Total mg/L	222	±75	340	±75	249	±70	283	±118
Mg Dissolved mg/L	720	±246	1078	±204	767	±219	955	±319
Mg Total mg/L	748	±253	1133	±2±51	826	±248	973	±350
Na Total mg/L	6306	±2046	9240	±1565	7047	±1822	7830	±2659

Table A1 Water parameters by lease including heavy metals, Water parameters for 5th to 12th February (source of data DPIWE 2004a)

Water parameter	n=17
1420-Water Dibutyltin (ng Sn/L)	<2.0
Tributyltin ng Sn/L	<2.0
a-BHC ug/L	<0.1
Aldrin ug/L	<0.1
Alphamethrin ug/L	<0.1
b-BHC ug/L	<0.2
Chlordane ug/L	<0.5
Chlorpyrifos ug/L	<0.1
d-BHC ug/L	<0.2
Diazinon ug/L	<0.1
Dieldrin ug/L	<0.2
Dimethoate ug/L	<0.1
Disulfoton ug/L	<0.2
Endosulfan I ug/L	<0.1
Endosulfan II ug/L	<0.4
Endosulfan sulphate ug/L	<0.1
Endrin ug/L	<0.1
Endrinaldehyde ug/L	<0.1
Ethylparathion ug/L	<0.1
Famphur ug/L	<0.1
g-BHC ug/L	<0.1
Heptachlor ug/L	<0.1
Heptachlor epoxide ug/L	<0.1
Hexachlorobenzene ug/L	<0.2
Malathion ug/L	<0.2
Methyl parathion ug/L	<0.1
p,p'-DDD ug/L	<0.1
p,p'-DDE ug/L	<0.2
p,p'-DDT ug/L	<0.1
Phorate ug/L	<0.2
Sulfotep ug/L	<0.1
Thionazin ug/L	<0.2
Spinosad ug/L	<0.1

Table A2 Pesticide levels in leases across Georges Bay / Moulting Bay 5th to 12th February 2004 (source of data DPIWE 2004a)

	Date	
	14/05/2003 (n=1)	5/10/2005 to 9/11/2005 (n=4)
Pacific oysters size (mm)	90-100	not available
Arsenic Inorganic (mg/kg)	0.26	
Total Arsenic (mg/kg)	1.53	3.3 ± 0.7
Cadmium (mg/kg)	0.1	0.1
Chromium (mg/kg)	0.6	0.5 ± 0.4
Cobalt (mg/kg)	<0.1	<0.1
Copper(mg/kg)	3.1	5.6 ± 1.5
Iron (mg/kg)	47	46 ± 13
Lead (mg/kg)	<0.1	0.09
Manganese (mg/kg)	2.2	4.3 ± 1.5
Mercury (mg/kg)	<0.2	0.019
Nickel (mg/kg)	1.3	1.3 ± 0
Zinc (mg/kg)	53	3 ± 0

Table A3: Heavy metal assay of oyster meat from leases in Georges Bay / Moulting Bay as part of PSQAP monitoring during 2003 and 2005 (source of data TSQAP)

	Subtidal lease B Western Moulting Bay	Intertidal lease B Western Moulting Bay	Intertidal lease A Western Moulting Bay	Mouth of George River	Subtidal lease D Eastern Georges Bay
Variable	Vertical net tow	Horizontal net tow 10m over seagrass	Horizontal net tow 10m over seagrass	Horizontal net tow	Vertical net tow
Depth	5m	0.5m	0.5m	2m	10m
Tide	Dead low	Dead low	Dead low	Dead low	not recorded
Temp (degrees Celsius)	19.5	20.5	20	10.1	19
Salinity	28.7	27	27.6	18.3	14.7
	Diatoms	**Diatoms (very sparse)**	**Diatoms**	**Diatoms (very sparse)**	**Diatoms**
	Amphora sp.	*Navicula sp.*	*Guinardia sp.*	*Navicula sp.*	*Amphora sp.*
	Guinardia sp.	*Nitzchia closterium*	*Navicula sp.*	*Pleurosigma*	*Navicula sp.*
	Leptocylindrus danicus	*Pleurosigma*	*Pleurosigma*		*Pleurosigma*
	Navicula sp.				*Coscinodiscus sp.*
	Nitzchia closterium		**Dinoflagellates**	Jellyfish	
	Pleurosigma		*Ceratium fusus*		**Dinoflagellates**
			Prorocentrum rathymumn		*Gymnodinium sp.*
	Dinoflagellates				**Gyrodinium lachryma**
	Ceratium furca				*Noctiluca scintillans*
	Ceratium fusus				*Ostreopsis sp.*
	Dinophysis fortii				*Protoperidinium sp.*
	Noctiluca scintillans				*Prorocentrum gracile*
	Ostreopsis sp.				
	Protoperidinium sp.				
	Prorocentrum gracile				
	Prorocentrum rathymumn				
	Flagellates				
	Dictyocha speculum				

Table A4: Algology for 5th February 2004 (source of data, DPIWE 2004a)

	Intertidal lease A Western Moulting Bay	Intertidal lease D Eastern Georges Bay	Medea Cove Bridge	Tidal windrows
Variable	Horizontal net tow 10m over seagrass	Horizontal net tow 10m	Horizontal net tow 10m over seagrass	
Depth	1.0m	0.5m	0.5m	
Tide	not recorded	not recorded	incoming	
Temp (degrees Celsius)	19.4	20.2	21	
Salinity	25.7	23.2	22.1	
	Diatoms	**Diatoms**	**Diatoms**	**Dinoflagellates**
	Amphora sp.	*Navicula sp.*	*Navicula sp.*	*Noctiluca scintillans*
	Guinardia sp.	*Dinoflagellates*		
	Navicula sp.	*Ceratium fusus*		
	Pleurosigma	*Noctiluca scintillans (abundant)*		
	Coscinodiscus sp.			
	Dinoflagellates			
	Prorocentrum gracile			

Table A4 continued with additional sample sites

Lease type in Moulting Bay	intertidal	subtidal	subtidal	subtidal	intertidal
Date	17/10/2005	12/10/2005	02/11/2005	16/11/2005	14/11/2005
Depthm (m)	0.5	10	4	4	0.5
Method of Sampling	Bottle	Integrated	Integrated	Integrated	Bottle
Tide	0.9 low	4 hours in Flood	4hr flood	Outgoing	High
Ceratium_furca		3800	0		
Ceratium_fusus		1900	0		
Chaetoceros_affinis					15000
Chaetoceros_danicus	22000				3800
Chaetoceros_debilis		23000		530000	950000
Chaetoceros_decipens				130000	100000
Chaetoceros_lorenzianus		13000			21000
Chaetoceros_peruvianus				1900	
Chaetoceros_pseudocrinitus		25000			
Chaetoceros_radicans		34000			
Chaetoceros_socialis					5700
Dictyocha_speculum					1900
Dinophysis_acuminata		95			
Ditylum_brigthwellii				1900	1900
Eucampia_zodiacus		1300		93000	44000
Guinardia_delicatula				9500	
Guinardia_flaccida		9500		11000	59000
Gymnodinium_catenatum		2100			
Leptocylindrus_danicus					
Leptocylindrus_mediterraneus	6000	100000	0	110000	36000
Navicula_sp		1900			
Nitzschia_closterium					
Nitzschia_sp					1900
Proboscia_alata				9500	1900
Protoperidinium_sp			0	1900	
Pseudo_nitzschia_seriata_group	4100	6600			1000
Psuedo_nitzschia_delicatissima_group	20000	120000	7000	140000	66000
Rhizosolenia_fallax		7600			
Rhizosolenia_setigera		1900			
Skeletonema_costatum	160000	28000			
Striatella_sp		1900	0		
Thalassionema_nitzschoides		7600			
Thalassiosira_gravida	0				3800

Table A5: Algology for October and November 2005 in Moulting Bay, the unit is algal cells per ml (source of data, Analytical Services Tasmania report to TSQAP)

Histopathological findings	2003 (n=0)	2004 (n=178)	2005 (n=288)
Kidney			
Expanded intercellular spaces, intracytoplasmic vacuolation of epithelia cells or dilated tubules		49↑(31)	25↓(43)

Histopathological findings	2003 (n=63)	2004 (n=178)	2005 (n=288)
Stomach and intestines			
Expanded intercellular spaces in stomach and intestines walls and intracytoplasmic vacuoles and haemocyte infiltrate into wall	0↓ (5)	46↑(16)	0↓(25)
Digestive glands			
Atrophied digestive gland tubules lined by low cuboidal cells, expanded extracellular spaces / tubular necrosis	0↓ (10)	90↑(30)	0↓(49)
Gonad			
Expanded intercellular spaces and haemocyte infiltrate of gonad / ova necrosis	0↓(2)	15↑(5)	0↓(8)
Post spawn	0↓(5)	47↑(17)	0↓(24)
Mantle			
Multifocal erosion of the mantle	0↓(11)	96↑(32)	0↓(52)
Interstitium			
Expanded intercellular spaces of the interstitium / necrosis of leydig cells with occasional haemocyte infiltrate	0↓(11)	94↑(32)	2↓(52)

Table A6
The numbers of Pacific oysters from lease groups with histopathological findings during 2004 flood event. Arrows indicate if observed values are above or below expected values. The numbers listed below are total numbers for each lease group and combine grades 1to 3 histopathological changes.The expected values are in parentheses. The expected values are calculated from χ^2 analysis of year groups for each histopathological finding.

Histopathological findings	Intertidal Western Moulting /Georges Bay (Leases A-C) (n=109)	Intertidal Eastern Georges Bay (Leases D & E) (n=37)	Sub tidal Central Georges Bay (Leases F & G) (n=32)
Kidney			
Expanded intercellular spaces, intracytoplasmic vacuolation of epithelia cells or dilated tubules	44↑(30)	0(10)	5↓(9)
Stomach and intestines			
Expanded intercellular spaces in stomach and intestines walls and increased numbers of intracytoplasmic vacuoles and haemocyte infiltrate into wall	16↓(28)	0↓(10)	30↑(8)
Digestive glands			
Atrophied digestive gland tubules lined by low cuboidal cells, expanded intercellular spaces / tubular necrosis	53↓(55)	27↑(19)	10↓(16)

Table A7: The numbers of Pacific oysters from lease groups with histopathological findings during 2004 flood event. Arrows indicate if observed values are above or below expected values. The numbers listed below are total numbers for each lease group and combine grades 1to 3 histopathological changes.

The expected values are in parentheses. The expected values are calculated from χ^2 analysis of lease groups for each histopathological finding.

Histopathological findings	Intertidal Western Moulting /Georges Bay (Leases A-C) (n=109)	Intertidal Eastern Georges Bay (Leases D & E) (n=37)	Sub tidal Central Georges Bay (Leases F & G) (n=32)
Gonad			
Expanded intercellular spaces and haemocyte infiltrate of gonad / ova necrosis	1↓(9)	0↓(3)	13↑(3)
Post spawn	0↓ (22)	37↑(8)	0↓ (7)
Mantle			
Multifocal erosion of the mantle	70↑(59)	9↓(20)	17(17)
Interstitium			
Expanded intercellular spaces of the interstitium / necrosis of leydig cells with occasional haemocyte infiltrate	56↓ (58)	13↓(20)	25↑(17)

Table A7: The numbers of Pacific oysters from lease groups with histopathological findings during 2004 flood event. Arrows indicate if observed values are above or below expected values. The numbers listed below are total numbers for each lease group and combine grades 1to 3 histopathological changes.

The expected values are in parentheses. The expected values are calculated from χ^2 analysis of lease groups for each histopathological finding.

Chapter 2

Biochemical and histopathological changes in oysters in response to low salinity stress

INTRODUCTION

Oysters are robust aquatic bivalve molluscs that adapt to highly variable inter-tidal and estuarine environments (Galtsoff 1964) and similar to other marine bivalves in this ecosystem they tolerate a broad range of salinities and temperatures (Dame 2012). Oysters are euryhyaline and osmoconform to gradual changes in salinity (Kinne 1964) (Gullian and Aguirre-Macedo 2010) which allows oysters to adapt to a broad range of temperatures and salinities associated with seasonal change. However, oysters cannot adapt quickly to rapid changes in salinity while experiencing elevated environmental temperature in spring or summer, when they have a high metabolic rate (Galtsoff 1964; Shumway 1996). Oysters will respond to unfavourable environmental conditions, like other bivalves such as mussels, by initially shutting their shells (Davenport 1981), but if the fall in salinity is marked and persistent, feeding and respiration will cease and mortalities can occur (Galtsoff 1964). Significant oyster mortalities were recorded in Georges and Moulting Bays, north eastern Tasmania, Australia, after severe freshwater flooding (1 in 50 year event) in February 2004 (summer), with up to 90% oyster stock losses in leases closest to the river mouth as it flowed in to Georges Bay (DPIWE Tasmania 2004).

An additional source of stress for farmed oysters, aside from environmental factors such as raised temperatures, occurs during and after grading (Lacoste et al. 2001; Li and Vanderpeer 2002; Percival and Ellard 2004; Zhang and Li 2006). Grading involves removing oysters from the water and subjecting them to mechanical rumbling and automated size sorting along a stainless trough or barrel (Zhang and Li 2006). Grading is an integral part of stock management, which facilitates faster oyster growth rates, because oysters appear to grow better when similar sized animals are grown together in baskets or trays, and it also encourages new shell growth by removing old shell (Zhang and Li 2006). Tasmania oyster growers often report variable stock losses following grading, particularly when they are associated with other stress factors such as high rainfall and freshwater flooding (Batley et al. 2010). Oysters' physiological responses to grading include abrupt spikes in haemolymph catecholamines (Lacoste et al. 2001) and decreased haemocyte

phagocytosis, the latter can lead to lower host protection against pathogens (Zhang and Li 2006).

Alongside elevated temperature, and management factors such as grading, genetic oyster traits are potential predisposing risk factors associated with freshwater oyster mortality events (Percival and Ellard 2004). Survival attributes are more heritable than other commercial selection traits (Evans and Langdon 2006), such as growth and condition, which are common selection traits for farmed oysters (CSIRO 2002) (CSIRO 2002). For these reasons family lines may respond differently to freshwater stress, just as some family lines had better survival rates than others during summer mortality events in France (Huvet et al. 2010).

Histopathology, is commonly used as part of oyster health surveillance programs, disease diagnosis, mortality investigations (Ellis et al. 1998; DPIWE Tasmania 2004 b; Kim and Powell 2006) and for bio-monitoring (Kim and Powell 2007) because it has potential to explore the interaction of oysters with pathogens or the environment (Yevich and Yevich 1994; Grizel 2003; Myers and McGavin 2007; Berthe 2008; Kim and Powell 2009). In the absence of infectious pathogens, water data and physiological changes are important for interpreting microscopic changes (DPIWE Tasmania 2004a; Bignell et al. 2008; Kim and Powell 2009). For these reasons, additional information, including water quality data on oyster leases and, where practical physiological biochemical parameters contribute to interpretation of histopathological changes, particularly following mortalities (Bignell et al. 2008). The aim of this study was to explore the biochemical and histopathological response of Pacific oysters (*Crassostrea gigas*) to an abrupt fall in salinity.

MATERIALS AND METHODS

Experimental Design and Setup

A three factor orthogonal experimental design was used to examine differences in histopathology of Pacific oysters (*Crassostrea gigas*) in response to water salinity (normal salinity 35ppt and low salinity 9ppt), grading (graded and ungraded oysters), and breeding (two family lines). Salinity and grading were fixed factors, while family line was a random factor (Table 1). Three replicate tanks (3 x low salinity 9ppt and 3

x normal salinity 35ppt) were used for every combination of the three factors. A salinity of 9ppt was used in this experiment because it is at the lower end of the mesohyaline range for oysters and we wished to examine for reversible and irreversible histopathological changes in oysters, not mortalities in response to salinity.

		Family A		Family B	
		Graded	not graded	Graded	not Graded
Salinity ppt	35	10	10	10	10
	9	10	10	10	10

Table 1: The orthogonal design for both the summer and winter experiments. The numbers of oysters for each factor (family, grading, salinity). Tanks were run in triplicate, that is, there were 3 tanks at 9ppt and another 3 at 35ppt with the same combination of oysters from the respectively families or history of grading.

The experiment was run twice, once in summer (February 2010) and once in winter (July 2009) to determine if the oyster response depended on water temperatures. Pacific oysters were collected from a commercial oyster farm in north-western Tasmania and sent by overnight courier to the Animal Health Laboratory DPIPWE Tasmania, Launceston. In summer the oysters were sent with a small shipment of ice bricks to ensure they did not overheat during transport. Ice bricks were not required for transport during winter. The oysters were transferred to the experimental tanks (at salinity either 9ppt or 35ppt) on the day they arrived at the laboratory. In the winter experiment family lines YC06-22E and YC06-4A were used, while the summer experiment used lines PI 1 and PI 3. Different family lines were used because the family lines used in the winter experiment were all harvested and unavailable for the summer experiment. The mean length of Pacific oysters for the winter experiment was 97 ± 8 mm (mean ± SD, n=80) and for the summer experiment 75 ± 6 mm (n=77). Half the oysters from each family line were graded at the farm before being sent to the laboratory.

At the oyster lease in north-western Tasmania the water temperature and salinity were recorded by the farmer using an alcohol thermometer and refractometer

(Vitalsine, Model SR-6) respectively, on the day the oysters were collected. On the day of collection for oysters for the winter experiment surface water salinity was 35ppt and temperature was 8^{o}C so water temperature in tanks was maintained at 8^{o}C and the control tanks had salinity at 35ppt. For the summer experiment, on the day of collection, surface water salinity was 35ppt and temperature was 18^{o}C on the oyster lease so water temperature was maintained at 18^{o}C in all tanks and control tanks had salinity at 35ppt.

Two independent re-circulating seawater systems were set up. Each system was held at either salinity 9ppt or 35ppt. De-ionised water was used to dilute the 35ppt seawater to 9ppt. Each seawater system had three 60L tanks and a biofilter with aeration provided by movement of water through the system and a water stone in each tank. Daily water ammonia/ammonium was tested with an NH_3/NH_4 API (R) test kit and water temperature was measured three times daily with an alcohol thermometer. Ammonium / ammonia levels were maintained at or below 0.25mg/L through daily partial water changes. Salinity was measured daily using a refractometer (Vitalsine, Model SR-6). In each of the six tanks, four oyster baskets were suspended, containing 13-14 oysters, which were from one of the four combinations of family line and grading. The oyster farm provided three fewer oysters for the summer experiment than for the winter experiment.

The experiment ran for 10 days and 40 oysters were randomly sampled on Day 3 and another 40 on Day 10, from each treatment group. It was decided not to feed oysters during the 10 day experiment as the microalgae usually used to feed Pacific oysters in culture would have lysed in the 9ppt salinity tanks. Oyster shells were examined for abnormal conformation, shape and defects (e.g. fluting) at the beginning of the experiment and when sampled on Days 3 and 10. Each oyster was opened by removing the flat shell valve and examined for the presence of gross lesions in the oyster meat and the colour, distribution, pattern, shape, contour, size, organ or site and change in texture of any lesions was recorded. From each oyster pallial cavity 0.2-0.4ml of fluid (free water in the closed oyster shell) was collected using a single use disposable plastic 1ml pipette and 0.2-0.4ml of haemolymph, from

the pericardial sac, was collected using a 1ml syringe and 21G needle (Becton Dickson (R)). Then the whole oyster was fixed in 10% seawater buffered formalin.

Analytical methods and histopathology

Concentrations of sodium and potassium in the oyster haemolymph were determined using a Konelab (R) automated biochemical analyser. Samples were diluted 1 in 2 or 1 in 3 with distilled water so concentrations were not above the limit of detection of the Konelab. Pallial cavity fluid salinity was measured using a refractometer (Vitalsine, Model SR-6), and pH was measured with a pH meter (MiniLab pH meter with ISFET solid state sensor, model IQ125 manufactured by IQ Instruments, Carlsbad, California, USA).

The formalin fixed tissues (preserved in 10% buffered saline formalin) were embedded in paraffin using standard techniques, cut at 5µm thickness and stained with haematoxylin and eosin. All the slides were read by one pathologist and each organ or anatomical site (kidney, heart, mantle, interstitium, gonad, ganglia, gill, stomach, intestine, digestive gland) of the oyster was examined. Histopathological changes were recorded and graded using a four point grading scale; 0=normal tissue and no microscopic changes, 1=mild changes with minor alteration to organ architecture, 2=moderate changes that affected and/or disrupted > ½ of the organ architecture, 3=severe changes with marked disruption or effacement of the majority of the organ architecture. A subset of 20 slides was independently read by a second pathologist to confirm reproducibility of results and uniform interpretation. The agreement between descriptions and grades was measured as the percentage of descriptions and grades given by the second pathologist who correlated with the descriptions and grades of the author.

Statistical analyses

Differences in mean haemolymph potassium and sodium concentration, pallial cavity fluid salinity and pH were examined as a function of salinity (normal or low salinity), grading (graded or ungraded), family line, season, and sampling day (Day 3 and 10) using a factorial ANOVA. Assumptions of homogeneity of variance were tested using

Levene's Test and data were log transformed where necessary. Significant sources of variability were examined with “a posteriori” Tukey’s HSD test.

Chi–squared (χ^2) test of independence was used to determine if the relative frequency of oysters with histopathological changes for each organ or anatomical site differed as a function of salinity (normal or low salinity), grading (graded or ungraded), season (winter or summer water temperature), family line and day sampled (day 3 and 10). The test of independence assumed that the number of individuals in the different histopathological categories were the same for all treatments. If the χ^2 analysis was significant the standardised difference between the expected frequency and the observed frequency was used to identify where differences had occurred. All tests were conducted at significance level <0.05 and all data were analysed using SPSS v18 (R).

In the winter experiment one ungraded oyster at normal salinity sampled on day 3 had insufficient haemolymph in the pericardial sac for testing. During the summer experiment in a low salinity tank one ungraded oyster died at day 5 and results from this animal were not included in the statistical analysis.

RESULTS

At the beginning of winter and summer experiments there was no evidence that any of the oysters in the experiments had defects or abnormalities in their shells and there was no chipping or break of oyster shell seals in either graded or ungraded oysters. In both experiments on Days 3 and 10 there were no gross abnormalities or lesions evident.

The factors grading and family lines did not contributed to statistically significant variability in any of the response variables ($P > 0.05$).

Mean pallial cavity salinity differed between groups of oysters that experienced different salinity and was dependent on season and day of sampling ($F_{(season*salinity*day)}=62.670$, df 7,147; $P<0.001$). Winter experiment oysters held at

salinity 9 ppt had mean pallial cavity salinity by day 10 that was 21.0% below the mean for oysters held at 35 ppt (Table 2). In contrast, in summer mean pallial cavity salinity in oysters held at salinity 9 ppt at day 3 and 10 were 56.3% and 65.6% below that of the oysters held at 35 ppt. All these differences in mean values for pallial cavity salinity were statistically significant based on “a posteriori” testing (Tukey’s HSD)

Similarly mean hemolymph sodium ($F_{(season*salinity*day)}$=170.373, df 7,147; P<0.001) and hemolymph potassium ($F_{(season*salinity*day)}$=62.670, df 7,147; P<0.001) differed between groups of oysters that experienced different salinity and was dependent on the season and day of sampling. In the winter, by day 10, mean hemolymph sodium and potassium of experimental oysters held at 9 ppt were 23% and 21.0%, respectively, below the mean for oysters held at 35 ppt (Table 2). In summer, mean hemolymph sodium in oysters held at salinity 9 ppt at day 3 and 10 were 63.7% and 72.3% below that of the oysters held at 35 ppt. Similarly mean hemolymph potassium for oysters at salinity 9 ppt on day 3 and 10 was 47.5% and 63.7% less than oysters at salinity 35 ppt.)

In summer and winter hemolymph pH was on average 7.3 with a range of 7.2 to 7.4 and there was no evidence that the variability in pH among the oysters was explained by any of the experimental factors (all terms in ANOVA had P > 0.05).

Variable	Winter				Summer			
	Water salinity 9 ppt		Water salinity 35 ppt		Water salinity 9 ppt		Water salinity 35 ppt	
	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=19)	Day 10 (n=17)	Day 3 (n=20)	Day 10 (n=20)
Pallial cavity salinity (ppt) mean ± SE	29 ± 0.4[a]	22.7 ± 0.4[b]	29.4 ± 0.4[a]	28.7 ±0.4[a]	15.4 ± 0.7[d]	13.1 ± 0.7[d]	35.2 ± 0.5[c]	38 ±0.7[c]
Haemolymph potassium (mmol/L) mean ± SE	10 ± 0.4[e]	8.5 ± 0.4[f]	9.5 ± 0.4[e]	10.7 ± 0.4[e]	6.3 ± 0.4[h]	4.5 ± 0.4[h]	12.0 ± 0.4[g]	12.4 ± 0.4[g]
Haemolymph sodium (mmol/L) mean ± SE	347.1 ± 4.6[i]	263.7 ± 4.6[j]	350.5 ± 4.7[i]	344 ± 4.6[i]	170± 15[l]	134 ± 15[l]	468.1 ± 15[k]	484.4 ± 15[k]

Table 2 Oyster pallial cavity salinity and haemolymph potassium and sodium results due to season, salinity and day of sampling. For each of the three variables, treatment means with different letters are significantly different from one another

There were microscopic changes in the stomach and intestines (χ^2=80.896, df 7; P<0.001), digestive gland tubules (χ^2=94.883, df 7; P<0.001) and kidney (χ^2=20.741, df 7; P=0.004) in oysters and the frequency of these changes was associated with salinity, season and day of sampling (Table 3 & Appendix Table A1). These microscopic changes in oysters (which were either grade 1 or 2; no grade 3 changes were seen) were more common in low salinity compared to normal salinity and this was particularly the case in summer. Expanded intercellular spaces, intracytoplasmic vacuoles in epithelial cells (taking up greater than 70-80% of the cytoplasm and moderately expanding the cell) and hemocyte infiltrate (diapedesis) in the walls of the stomach (Fig. 2), digestive gland tubules (Fig. 4), and intestines (all grade 1 to 2 for each organ) were observed. In the kidney there were expanded intercellular spaces in tubules and intracytoplasmic vacuoles in epithelial cells (taking up greater than 70-80% of the cytoplasm and moderately expanding the cell) lining the renal tubules (grade 1 to 2) (Fig. 6).Oysters in winter at low salinity had only limited microscopic changes in the kidney. These changes were mild (grade 1) intracytoplasmic vacuoles in renal cells and intercellular expanded spaces in the kidney and other kidneys showed no microscopic findings (Fig. 5). There were no severe (grade 3) changes in any organs. As the frequency of grades 1 and 2 for histopathological changes did not differ between treatment groups based on salinity, season, day, family or grading (P > 0.05), grades 1 and 2 were pooled for each histopathological description (Table 2).Oysters in winter at normal salinity showed no microscopic changes in the alimentary tract (normal stomach, Fig 1 and normal digestive gland, Fig. 3). Family line and grading did not affect the frequency of histopathological changes due to low salinity in either summer or winter.

The most significant histopathological findings from the oyster which died in low salinity in the summer experiment were mild (grade 1) diffuse infiltrate of haemocytes into the wall of the stomach, intestines and digestive gland tubules (grade 2) and dilation of digestive gland tubules (grade 1). Haemolymph potassium was 13.8mmol/L and sodium was 136mmol/L, these were within a range to the other oysters at salinity 9ppt.

For 95% of slides reviewed by both the author and second pathologist there was agreement in descriptions and grades. For 5% of cases (2 cases in total) there was a discrepancy between grade 1 and 2 intracytoplasmic vacuoles in renal epithelium. All other histopathological findings for the oysters were in agreement. Given that grades 1 and 2 were pooled this was a minor discrepancy and the author's grade was agreed, after discussion with the second pathologist, to be included in the results for the research report.

	Winter				Summer			
	Water salinity 9		Water salinity 35		Water salinity 9		Water salinity 35	
Histopathological finding	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=19)	Day 10 (n=17)	Day 3 (n=20)	Day 10 (n=20)
kidney – expanded intercellular spaces and intracytoplasmic vacuolation	5↓	7↑	6	3↓	13↑	8↑	4↓	3↓
Digestive gland - expanded intercellular spaces intracytoplasmic vacuolation and haemocyte infiltrate	0↓	0↓	0↓	0↓	14↑	13↑	1↓	2↓
Stomach and intestines - expanded intercellular spaces, intracytoplasmic vacuolation and haemocyte infiltrate	0↓	0↓	0↓	0↓	11↑	10↑	0↓	0↓

Table 3: The number of oysters with histopathological changes in the kidney, digestive gland, stomach and intestines (combining grade 1 and 2) on day 3 and 10, in either low, 9 ppt, or normal, 35 ppt, salinity, for each season. Arrows indicate if observed values are above or below expected values (based on chi-squared analysis).

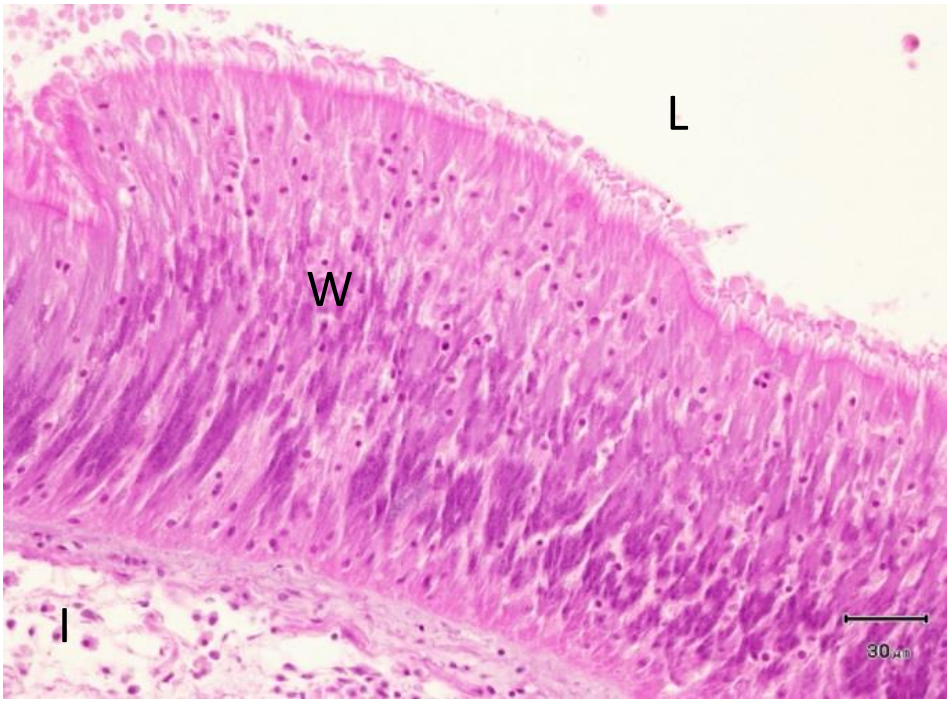

Figure 1: The normal stomach wall (W) consists of tall epithelial cells with small round nucleus set in the median part of the cell. The gastric lumen (L) and underlying interstitium (I) are labelled also

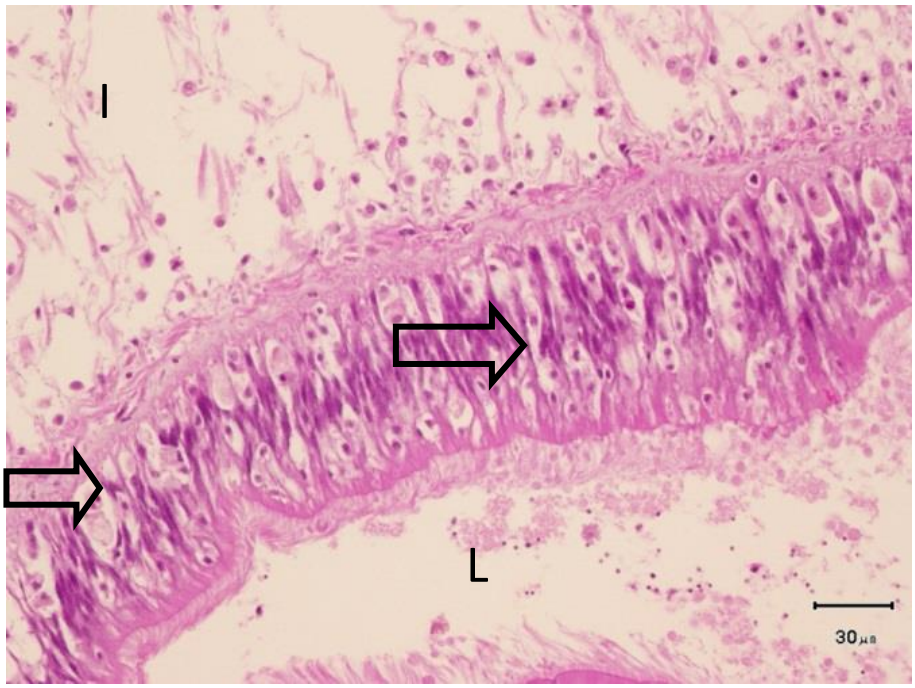

Figure 2: The stomach wall is moderately diffusely expanded by intercellular spaces (arrows) in low salinity, day 10 in summer

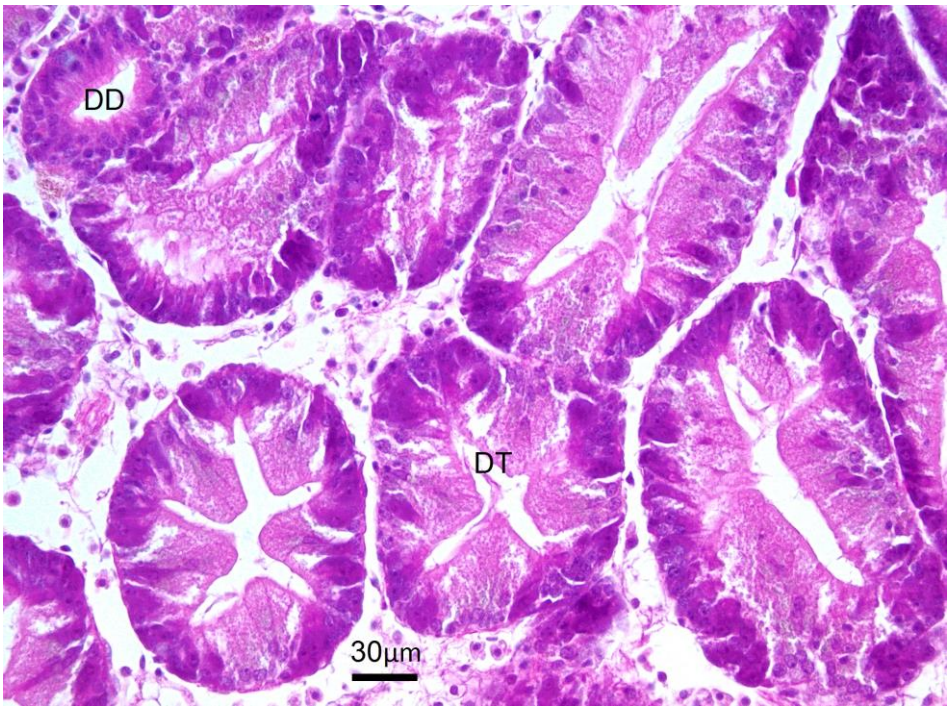

Figure 3: Normal digestive glands consist of digestive ducts (DD) and digestive tubules (DT).

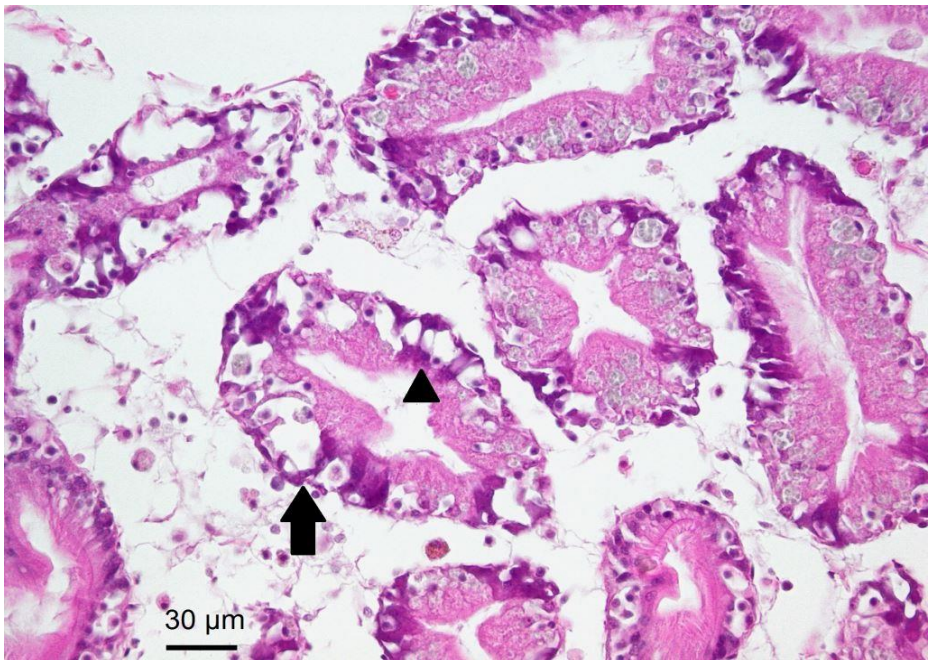

Figure 4 Expanded intercellular spaces, and haemocyte infiltrate (arrow) within the digestive tubular wall and intracytoplasmic vacuolation of epithelial cells (arrow head) in an oyster at low salinity, day 10 in summer

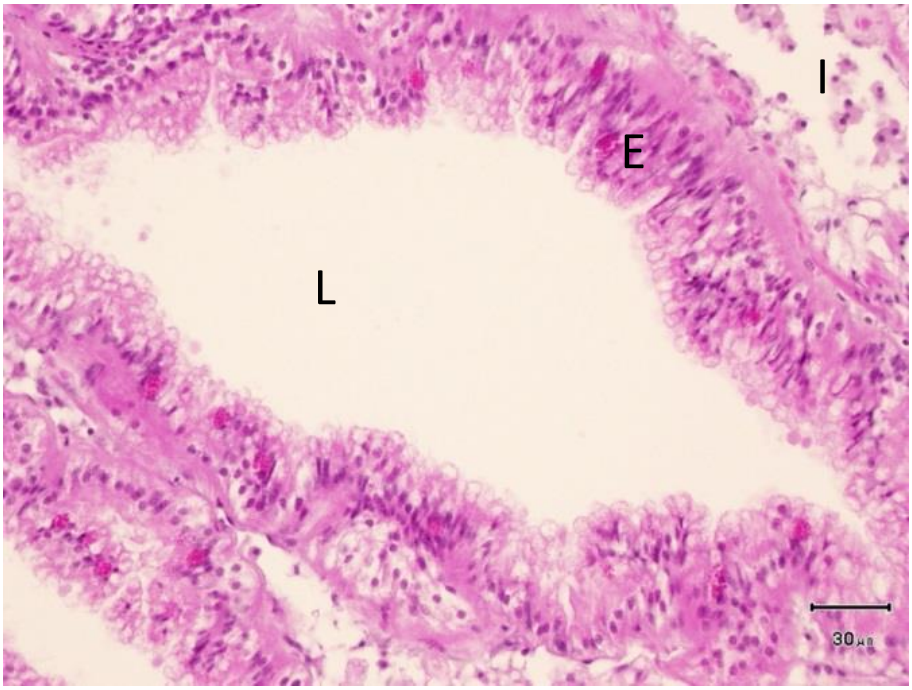

Figure 5: Normal renal tubule lined by closely packed columnar epithelium (E) with clear apical poles. There is a tubular lumen (L) and supporting interstitium (I).

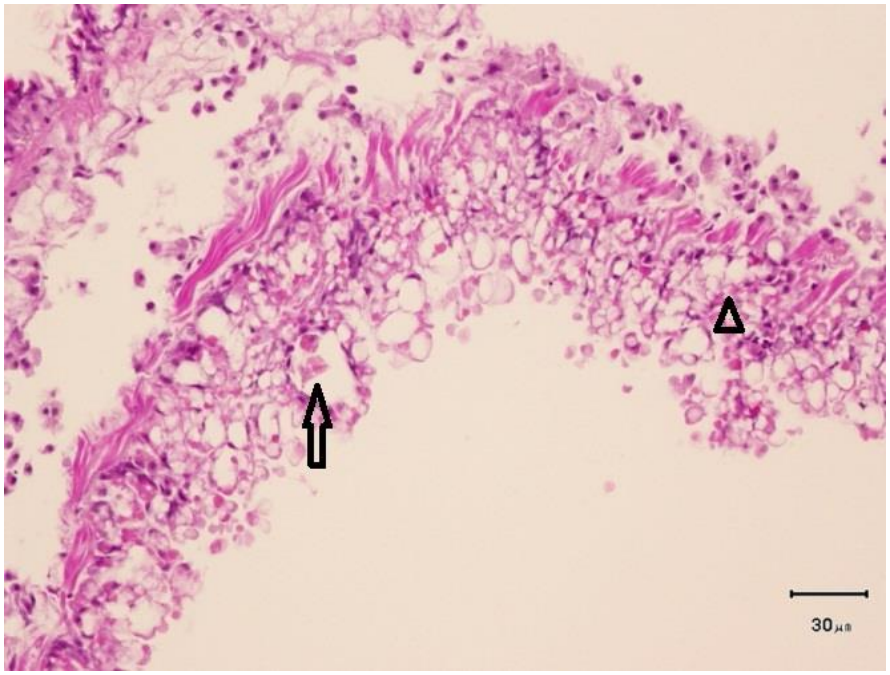

Figure 6 There are abundant intracytoplasmic vacuoles (arrowhead) and the intercellular spaces are expanded (arrow) in the kidney tubule (grade 1) from oyster at low salinity in summer, day 10

DISCUSSION

This experiment demonstrated that elevated summer temperature and low salinity working together were associated with adaptive renal and alimentary changes in osmoconforming oysters. This is consistent with the influential roles of temperature and salinity on physiological processes in oysters, such as Eastern oyster *(C. virginica)* (Galtsoff 1964, Shumway 1996).

In summer oysters at low salinity (9ppt) opened their shells and osmoconformed to low salinity sooner than those in winter. In the summer experiment increased respiratory demand at higher temperatures (Shumway and Koehn 1982) most likely over-rode the intracellular signals from sodium ion receptors (Natochin et al. 1979) to maintain the shells closed. Higher summer water temperature increases respiration rate and oxygen demand (While their shells are shut bivalves cannot feed or take in oxygen (Dame 2012). Warmer water temperatures increase respiration rate and oxygen demand in oysters (like other bivalves) (Shumway and Koehn 1982) and limit how long oysters can remain closed (Loosanoff 1953; Galtsoff 1964). In the summer experiment it is possible that increased respiratory demand at higher temperatures (Shumway and Koehn 1982) most likely over-rode the intracellular signals from ion receptors to maintain the shell closed (Natochin et al 1979). After bivalves open their shells, the extracellular fluid osmoconforms to the surrounding water ((Berger and Kharazova 1997). In the low salinity tanks in summer haemolymph sodium and potassium was lower in low salinity compared to normal salinity at day 3, as oysters opened their shells to low salinity water, 9ppt (Loosanoff 1953, Galtsoff 1964).

In winter the pallial cavity salinities in oysters held in 35ppt tanks were 29ppt and not 35ppt. If the oysters were fed and respired with an open shell the salinity in the pallial cavity would be the same as in the surrounding water. It is possible that pallial cavity salinity was 29ppt and not 35ppt because when and oysters were removed from their baskets in the water on the farm lease and sealed their valves the surrounding water was at salinity 29ppt, and this water was retained in the pallial cavity throughout the experiment. Records on the day, when oysters were collected from the farm, suggested that the salinity was 35ppt, however, localised variation in salinity on the

estuarine farm lease may explain this discrepancy in reported salinity. While it was expected that oysters at salinity 9ppt would remain shut, it was expected that animals in salinity of 35ppt would open and metabolise aerobically. However, the cool water temperature (8^{o}C) of the winter experiment, may have slowed metabolic rates such that animals did not need to open their shells for respiration (Loosanoff 1953), and over the ten days did not exchange their original of pallial cavity fluid with tank water and their cavity salinity did not conform to the 35ppt salinity in the tanks.

Accumulation of anaerobic metabolites is another reason why oysters, such as *C. virginica* and other bivalves such as *M. edulis*, cannot keep their shells closed (Zubkoff and Ho 1982) after persistent stressful environmental challenges such as low salinity. When bivalve molluscs are closed they accumulate anaerobic metabolic by-products such as succinate and fatty acids (Wijsman 1976; Zubkoff and Ho 1982) from the incomplete oxidation of glycogen for adenosine triphosphate generation through the tricarboxylic acid pathway (de Zwaan 1977). To extend the period they can remain shut under anaerobic conditions (closed shell) bivalves decrease their rate of metabolism (de Zwaan and Wijsman 1976; Hawkins and Bayne 1992; Hochachka and Somero 2002). In winter oysters at Day 3 in low salinity demonstrated this metabolic adaptability by remaining closed but with no significant decrease in pallial cavity fluid pH, suggesting decreased metabolic rate with minimal production of acidic metabolic by-products (de Zwaan 1977).

One of the key microscopic changes associated with low salinity and elevated summer temperature in osmoconforming oysters were expanded intercellular spaces and significantly vacuolated cells in the kidney. Similar changes have been reported in *Mytilus* sp. when challenged by abrupt low salinity (Khan and Saleuddin 1986). and when the remaining mussels were returned to seawater the renal changes resolved. Based on this evidence and the fact there was necrosis of renal cells or irreversible damage to renal cell integrity or intercellular structures (Myers and McGavin 2007), the expanded intercellular spaces and vacuolated renal cells in the Pacific oysters, during the experiment, may also be reversible.

Significant intracellular vacuolation distending epithelial cells was common in both renal and alimentary epithelial cells in oysters at low salinity. The roles of renal cells include low grade partial osmoregulation (along with the gills) of haemolymph, excretion of nitrogenous waste and phagocytosis and pinocytosis of excreted product through lysosomal intracytoplasmic vacuoles (Florey 1966; Grizel 2003). As low salinity and elevated temperature increased, osmotic stress and respiratory demand, may have increased not only the osmoregulatory role of the kidney (which it shares with the gills) but the demand and production of phagocytic lysosomal vacuoles. Similarly the significant intracytoplasmic vacuoles in the digestive gland may reflect the increased metabolic demand due to low salinity and increased temperature. Intracytoplasmic lysosomes are one of the key processes for digestion in bivalve molluscs (Owen 1972; Pal et al. 1990; Weinstein 1995). Stomach and intestines do not play a primary role in intracellular digestion however, vacuolation in osmoconforming oysters at summer may reflect the accumulation of breakdown metabolic products retained within lysosomal vacuoles for release into the lumens (Galtsoff 1964).

Pollution can also cause intracytoplasmic vacuolation. It should be noted that intracytoplasmic vacuolation of digestive glands were found in *Mytilus edulis* exposed to polluted waters in Tvarminne area Gulf of Finland (Baltic sea) (Sunila 1987), *Crenomytilus grayanus* exposed to polluted waters in Peter the Great Bay, Sea of Japan (Usheva et al. 2006) and *Mytilus edulis* exposed to metals such as copper and cadmium (Sarasquete et al. 1992). However, pollutants or heavy metals were unlikely to have contributed to the vacuolation in the experimental oysters in this trial because these oysters were from leases which were tested negative for heavy metals (including copper, zinc, cadmium, lead, aluminium) and pesticides (including Endosulfan, Tributyltin, Malathion) by water testing and oyster meat testing, overseen by the Tasmanian Shellfish Quality Assurance Program (TSQAP) for public health (A. Turnbull, TSQAP, pers. comm.). In addition distilled water, which is very unlikely to be the source of pollutants, which was unlikely to be the source of pollutants, was used in the trials to dilute the sea water.

Along with intracytoplasmic vacuolation of gastric and intestinal epithelial cells, there was mural transmigration of haemocytes, known as diapedesis (Onstad et al. 2006) (Jones 2010) between the expanded intercellular spaces in oysters at low salinity in summer. Diapedesis through the alimentary tract (and other organs) can be a normal finding in aquatic bivalve molluscs, such a *Mytilus edulis* (Sunila 1988; Onstad et al. 2006; Jones 2010). However, diapedesis was observed more commonly at low salinity in summer along with expanded extracellular spaces in the alimentary tract. Mural diapedesis through the alimentary tract wall can be due to pathogenic or benign environmental bacterial chemotactic factors (Cheng and Howland 1979). Diapedesis is also seen when heavy metals are transferred to the alimentary tract lumen by haemocytes within intracytoplasmic tertiary lysosomes (George 1983). As discussed above, heavy metal contamination of the oysters or the water used in the experiment was very unlikely.

When assessing microscopic changes in oysters it is important to exclude, as far as possible, the contribution of infectious pathogens. In the experimental oysters there were no histopathological findings consistent with infectious diseases such as iridovirus (Elston and Wilkinson 1985), marteiliosis (Thebault et al. 2005) haplosporidiosis (Perkins 1968), perkinsiosis (Villalba et al. 2004) nocardiosis (Friedman 1990) and mikrocytosis (Hervio et al. 1996) and infection with *Bonamia* sp., (Hine et al. 2001) the latter is common in endemic Tasmanian *Ostrea angasi*. Ostreid Herpesvirus 1, a significant pathogen of Pacific oysters is rarely reported to cause pathognomonic intranuclear viral inclusions but can cause variable histopathological changes, such as haemocytes with pyknotic nuclei or fragmented nucleus, mantle epithelium necrosis or haemocytosis in the interstitium of digestive gland tubules (Friedman et al. 2005). None of these changes were seen in experimental oysters. Because a diagnostic test for Ostreid Herpesvirus was not available in Tasmania, the experimental oysters could not be tested free of OsHV1 but subsequently in 2011, 2012 and 2013 state-wide testing of all Pacific oyster growing areas in Tasmania, conducted by DPIPWE Tasmania in conjunction with the Tasmanian Oyster Research Council demonstrated no evidence of OsHV1 by PCR testing (Ellard 2011, 2012, 2013.). In summary, infectious pathogens were not identified on histopathology, which could have contributed to the microscopic

changes. Taking into account OsHV1 has not to date been detected in farmed Tasmanian Pacific oysters, OsHV1, also is unlikely to have contributed to the microscopic findings in the experimental oysters.

In this experiment there was no evidence that differences among the variables measured were attributable to the oysters' breeding history or their exposure to grading stress just prior to arriving at the laboratory for the experiment. They did not appear to influencing the time oysters opened (change in pallial cavity salinity) osmoconformed or the microscopic changes associated with osmoconformation, at low salinity. Grading would directly affect the response to an abrupt change in low salinity if the shell seal was broken during grading (Loosanoff 1953). Although the oyster's mantle will cover any hole in the shell (Loosanoff 1953) the mantle is not an impermeable barrier and there should have been changes in pallial cavity salinity if the seals were broken during grading. Because there were no significant chips or breaks in the shell seals or valves after grading it appeared that the oysters had a complete seal when exposed to low salinity water and as a result grading had no effect. The oysters of all four families had a similar response to abrupt change to low salinity suggesting there is little significant genetic variation in this trait between the four family lines.

In summary the stress effects of elevated summer temperature and abrupt change to persistent low salinity caused reversible microscopic changes in the kidney and alimentary tract of osmoconforming oysters. Describing these microscopic changes will aid diagnosticians in their interpretation of molluscan histopathology. These results will also enable better management of stress events experienced by oysters in culture conditions and ultimately inform industry of the nature of mortality events due to environmental challenges.

Further research is required to compare these microscopic changes in oysters challenged at the lower end of mesohyaline salinity (9ppt) to oysters challenged by freshwater in summer.

Appendix

	Winter				Summer			
	Water salinity 9		Water salinity 35		Water salinity 9		Water salinity 35	
Histopathological finding	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=19)	Day 10 (n=17)	Day 3 (n=20)	Day 10 (n=20)
kidney – expanded intercellular spaces and intracytoplasmic vacuolation	5↓ (6)	7↑(6)	6(6)	3↓(6)	13↑(6)	8↑(6)	4↓	3↓
Digestive gland - expanded intercellular spaces intracytoplasmic vacuolation and haemocyte infiltrate	0↓(3)	0↓(3)	0↓(3)	0↓(3)	14↑(3)	13↑(3)	1↓(3)	2↓(3)
Stomach and intestines - expanded intercellular spaces, intracytoplasmic vacuolation and haemocyte infiltrate	0↓(3)	0↓(3)	0↓(3)	0↓(3)	11↑(3)	10↑(3)	0↓(3)	0↓(3)

Table A1. Histopathological effects of abrupt salinity changes at winter and summer. Arrows indicate if observed values are above or below expected values (based on chi-squared analysis). The numbers listed below are total numbers for each day and combined grade 1 and 2 histopathological changes. The expected values (values are round to the nearest whole number) are in parentheses.

DISCUSSION

This was a retrospective study of histopathological findings from the Georges Bay oyster mortality event February 2004, which were interpreted in the light of environmental water data, and compared to findings in oysters collected outside the period of the flood event. Because of the limited scope of this retrospective study, not all the interacting factors which caused the oyster mortality event could be investigated. Some key histopathological findings in oysters affected by the freshwater flooding included changes in the kidney and alimentary tract which were consistent with osmoconformation to the abrupt low water salinity recorded during the flooding. Experimental trials replicated these histopathological changes associated with abrupt fall in salinity and osmoconformation and the interactive effect of season (primarily elevated summer temperature) and day of sampling, on the oysters' response to low salinity.

The experimental findings from this research project are important because they demonstrate, through biochemical changes in haemolymph, and histopathology, pathophysiological host responses to abrupt fall to low salinity, such as during the February 2004 floods. In oysters from the Georges Bay and Moulting Bay mortality event in February 2004 there were markedly expanded intercellular spaces in the walls of the stomach, intestines and digestive glands, significant intracytoplasmic vacuolation and moderate diffuse intramural haemocyte infiltrate (diapedesis). In addition there were expanded intercellular spaces in the kidneys. All these changes were replicated in oysters exposed to abrupt change to low salinity in experimental tanks in summer.

Abrupt change to low salinity is a stress for bivalves, including oysters (Galtsoff 1964) and freshwater flooding, often when the water temperature is elevated, are associated with a number of mortality events of wild endemic bivalves, such as *S. corneus, Macoma litoralis* in Swatzkops estuary near Port Elizabeth in South Africa (McLachlan and Erasmus 1974), *Flaviolanatus subtorta* and *Notospisula trigonella* in the lower reaches of the Hawkesbury river of NSW, (Jones 1989), *Soletellina alba and Arhtritica helmsi* in mouth of the Hopkins River, Victoria, Australia (Matthews

and Constable 2004) , and *Pinctada imbricata* at the mouth of the Hastings River, NSW, Australia (O'Connor and Lawler 2004) and cultured oysters Eastern oysters (*C. virginica*) on the east coast of the USA (Gunter 1950; Andrews et al. 1959). Previous experimental trials replicated mortalities in adult *Soletellina alba* following abrupt change to low salinity (Matthews and Fairweather 2004) and changes in physiological behaviours, such as decreased pallial fluid pumping in Eastern oysters after abrupt change to low salinity (*C. virginica*) (Loosanoff 1953). However the experiments included in this thesis are one of the few studies, along with Khan and Saleuddin (Khan and Saleuddin 1986) which assessed microscopic changes and interpreted these in the light of physiological osmoconformation.

In oysters from the Georges Bay and Moulting Bay mortality event in February 2004, along with the findings described above in the alimentary tract (including markedly expanded intercellular spaces, haemocyte diapedesis in the walls of the stomach, intestines and increased vacuolation in the epithelial cells) there was epithelial necrosis. There was no significant cell necrosis evident in the alimentary tract of oysters in the summer experiment at low salinity. The absence of necrosis was consistent with the lack of a rise in potassium haemolymph level at low salinity in summer experiment. Elevated haemolymph potassium reflects cell rupture and death in molluscs (Turgeon 1976; Natochin et al. 1979). A possible reason for the lack of necrosis was that the oysters opened their shells a) by day 3 in summer and b) by day 10 in winter, when their metabolic rate was presumably lower than in summer.

Additional microscopic changes seen in oysters from Georges Bay 2004 and not in the experimental oysters at low salinity in summer or winter included, gonad necrosis, leydig tissue necrosis, adductor myositis and expanded extracellular spaces and haemolymph vessels in the mantle. There were no water data findings to confirm the causes for these changes but prolonged shell closure and high metabolic demand could have contributed to leydig tissue necrosis (Galtsoff 1964). The lack of explanations for other microscopic findings show how complicated inter-related, multiple freshwater factors are, and limitations of water data testing for identification of all lethal environmental factors which caused microscopic oyster host responses.

The causes of many oyster mortality events are complicated because there are often multiple interacting risk factors. A simplification is to group them roughly in to infectious and non-infectious mortality events. For both groups there will be external / environmental risk factors and host susceptibility which will lead to onset of disease and mortalities and in the case of infectious events, a pathogens which interact both with external/environmental and host factors. Nocardiosis in western Australian Pinctada maxima (Pass et al. 1987) and *C. gigas* on the west coast of the USA (Friedman 1990) and Ostreid Herpesvirus micro variant 1 (OsHV1 micro variant) in *C. gigas* in France (Schikorski et al 2011) are well known examples of infectious pathogens causing mortality events in farmed oysters. Host factors such as age, in some herpes virus outbreaks in France, spat were more susceptible than other ages of oysters, and environmental factors such as rising water temperature predispose to mortalities due to OsHV1 (Schikorski et al 2011). Mortalities related to flood events during spring, directly correlated to oyster losses by both location and time frame (spatial and temporal correlation), and are examples of a non-infectious process (Andrews et al. 1959, Burrell 1977).

For completeness, when investigating oyster mortalities, infectious pathogens should be excluded as risk factors by diagnostic tests. This was done by histopathological examination of oysters for this project. Environmental risk factors (non-infectious) were most likely responsible for the February 2004 mortality event because a) no oysters, which were examined histopathologically, from the Georges Bay flood event of 2004, had findings consistent with infectious pathogens and b) the oyster mortalities followed very quickly after the floods and c) losses were mainly around the western areas of the bays, closest to the George River mouth in intertidal and not subtidal leases (the latter were exposed some of the day to deeper waters where the salinity may have been higher than surface water). There were no histopathological findings consistent with infectious diseases such as Iridovirus (Elston and Wilkinson 1985), Marteiliosis (Thebault et al 2005) Haplosporidiosis (Perkins 1968), Perkinsiosis (Villalba et al 2004) Nocardiosis (Friedman 1990) Mikrocytosis (Hervio et al 1996) and endemic Bonamia sp. (Hine et al. 2001) which commonly infect Tasmanian *Ostrea angasi*. Ostreid Herpesvirus PCR was not available in Tasmania at the time of the mortality event and there were no archived samples available for

retrospective PCR testing. Nevertheless there is no evidence based on oyster mortality monitoring through the Tasmania Pacific Oyster Health Surveillance Program (which has been in place since 1994; pers. communications K Ellard) of Ostreid Herpesvirus 1 associated oyster mortality events in Tasmania. This was supported by state-wide surveys of all oyster growing regions of Tasmania in 2011, 2012 and 2013 for OsHV 1 by quantitative polymerase chain reaction testing (Ellard 2011, 2012, 2013).

Surveillance of oysters through testing, such a histopathology, and matching these changes with seasonal and climatic data is important because oyster production and oyster mortalities are influenced by multiple interacting environmental factors. This study demonstrated the interaction of season (water temperature) and salinity was important. In experimental trials, oysters in winter resisted the osmotic effects caused by change to low salinity longer because their shells were closed. Oysters in summer opened sooner and responded by osmoconforming. Seasonal factors, in particular water temperature, were major contributing factors for Pacific oyster (*C. gigas*) mortalities in France (Samain and McCombie 2008), Japan (Mori 1979) and USA (Washington State) (Cheney et al 2000). In Australia, mortalities of wild and farmed bivalves following freshwater flooding are more common in summer than in winter (DPIWE Tasmania 2004, Matthews and Constable 2004, O'Connor and Lawler 2004). Farmers commonly manage seasonal factors as part of their husbandry and production of oysters for market, for example moving Pacific oysters from inter-tidal to subtidal leases to minimise the effects of summer heat stress (White 2001). This research has identified that Tasmanian farmers should be more concerned about the risks associated with freshwater floods during summer than winter, for their farmed oysters.

Oysters are collected and examined by histopathology for oyster health surveys such as the Tasmania Pacific Oyster Health Surveillance Program (Ellard 2012, 2013). These long term studies have the capacity to compare and interpret histopathological changes in oysters from different regional populations in conjunction with seasonal and climatic trends. For example, physiological indicators such as digestive gland atrophy and reproductive stage in Eastern oysters (*C.*

virginica) showed temporal trends across the Gulf and East coasts of USA which correlated to climatic changes related to El Nino (Southern Oscillation index) and North Atlantic Oscillation index (Kim and Powell 2009). In a similar way oysters could potentially be used as sentinels for invertebrate populations and their response to climate change. This study demonstrated the correlation between short term microscopic changes in kidney and alimentary tract (such as expanded intercellular spaces and significant intracellular vacuolation) and abrupt change to low salinity, due to significant local rainfall. Similar localised, time specific climate data could in the future be compared at multiple sites across Tasmania over a year to assess trends related to rainfall patterns and river inflows and their effects on oysters in farmed estuaries. Because slides and paraffin blocks from histopathological surveys, such as from the “Tasmanian Pacific Oyster Health Surveillance Program” (Ellard 2011, 2012) and “Mussel Watch Program” in the USA (Kim and Powell 2009), are archived, they are accessible for retrospective reviews. This retrospective review of histopathological changes was a very useful and reliable tool in conjunction with environmental data to understand how oysters responded to historic events. In addition, future programed oyster sampling for histopathology, in conjunction with climatic data, specific to the location and time of oyster sampling, could be to monitor the response of invertebrate communities to changes in climate over time (Kim and Powell 2009). In addition multiplexed assays (such as multiplex PCRs) could be used to screen sentinel bivalves for infectious pathogens (Berthe 2008). All this information could then be combined with multi-layered environmental data for potential real-time health management at the ecosystem level (Berthe 2008).

By running a tank trial with a known stressor factor, abrupt change to low salinity, which was associated with the February 2004 flood event, the histopathology related to low salinity was clarified. This study offered another non-toxic (i.e. low salinity) cause for significant intracytoplasmic vacuolation of digestive glands, in addition to other reported causes in other bivalves such as M. edulis exposed to polluted waters (Sunila 1987, Usheva et al. 2006) or copper and cadmium (Sarasquete et al. 1992).

Temporal factors as well as spatial distribution of freshwater will influence the degree of decrease in salinity and molluscan response to low salinity (Loosanoff 1953, Khan

and Saleuddin 1986). For example, the minimum salinity at high tide and the number of consecutive days below a specific salinity threshold are significant risk factors for mollusc mortality events in the Ulla river estuary, Galicia northwest Spain (Parada et al. 2012). Unfortunately detailed salinity data, across multiple sites in Georges and Moulting Bays were not recorded during the mortality event of February 2004. Nevertheless there was a history of mildly uneven, but not dramatically different, distribution of salinity across Georges and Moulting Bays, with leases near the Barway (eastern Georges Bay) having generally slightly higher salinity than those near the river mouth (western Georges Bay) (Brown 1998). This difference may have been more marked during the flood. Osmoconforming histopathology findings varied across the lease groups with those intertidal leases nearer the George river mouth having more osmoconforming changes in both kidney and alimentary tract. For these reasons, during the February 2004 flood event, low salinity floodwater was most likely not evenly distributed throughout the Georges and Moulting Bays. A recommendation from this research is that Tasmania oyster farmers, researchers and regulatory officers who investigate future freshwater flooding events, should take extensive salinity readings including each different lease site in an estuary, and at both low and high tide.

This study did not identify all interacting factors which caused the oyster mortalities in 2004. Because only histopathological changes were assessed in oysters from the February 2004 flood event, environmental factors which did not cause histopathological changes, such as abrupt falls in dissolved oxygen (which is associated with freshwater flooding and eutrophication), could not be assessed. In addition the tank experiments were not mortality experiments and for this reason, abrupt low salinity cannot be confirmed as the sole cause for the mortalities during the flood event of February 2004.

Having identified the effect of abrupt low salinity and water temperature (summer) as interacting stress factors, the next step will be to identify other stress factors which may also have occurred during the flood event of 2004 in Georges and Moulting Bays. The oysters closest to the mouth of the George River (in Western Georges and Moulting Bays) along with osmoconformation changes in the alimentary tract

and kidney, had a higher rate of mantle erosion. One possible cause for the mantle erosion was direct injury to the mantle by suspended sediment washed down with the flood waters (Shumway 1996). Suspended sediment initially stimulates oysters to close their shells and protect the fragile mantle and gills but once oysters re-open their shell physical damage to the mantle and gills can be extensive (Shumway 1996). Damage to the mantle will disrupt physiological functions such as osmoregulation and feeding and multifocal mantle necrosis was a characteristic finding in pearl oyster (*Pinctada maxima*) mortalities (Jones et al. 2010). For these reasons trials to assess histopathological changes in response to various sizes of suspended sediment on oyster mantle will help to clarify the effect of sediment on oyster mortalities during flood events.

The experimental tank trials correlated microscopic changes with osmoconformation but did not investigate physiological changes at the molecular level. Assessing gene expression for key adaptive processes designed to protect bivalves against osmotic shock, such as intracellular protein transport and synthesis and cell membrane ion channel activation, in conjunction with the microscopic changes in alimentary tract and kidneys will help to better understand the physiological response to abrupt change to low salinity on oysters. For example *M. edulis* and *M. galloprovincialis,* which are relatively susceptible to low salinity stress and better suited to stable salinity environments, up regulated genes associated with cell membrane ion channels (Kcna10), and amino acid transport (Slc17a5, Glyt2, Atp1a and Mct) when challenged by abrupt low salinity. These are both key components for protecting cell integrity in changing salinity (Boutet et al. 2005; Lockwood and Somero 2011). By comparison *M. trossulus*, which better adapts to fluctuating salinities or persistently low salinities, down regulated protein synthesis genes, such as ornithine decarboxylase (Odc), and up regulated translation genes, e.g. eukaryotic initiation factor 4A-III (Eif4a3), eukaryotic peptide chain release factor subunit 1 (Erf1) (Lockwood and Somero 2011), when challenged by abrupt low salinity. In a similar way understanding which genes are expressed by farmed Pacific oysters challenged by low salinity, will help to explain pathophysiology of mortality events and also offer potential non-lethal tissue biopsy testing for gene expression monitoring as part of a process to predict mortality events (Cheney and Gracey 2011).

Summary

The retrospective assessment of histopathological findings from the Georges Bay oyster mortality event, 2004, in light of environmental data and oysters collected outside the period of the flood event identified osmoconforming changes in the kidney and alimentary tract, as key findings, which were supported by water salinity readings. Experimental trials replicated these histopathological changes associated with abrupt fall in salinity and osmoconformation and the interactive effect of season (primarily elevated temperature and spawning associated with summer) on the oysters' response to low salinity. Recommendations from this work are that extensive salinity and dissolved oxygen readings, where practical, be taken at multiple lease sites across flooded estuaries during any future oyster mortality events. These should be supplemented by extensive water testing, similar to the range of tests taken during the Georges and Moulting bay flood event of 2004. Those oysters closest to the river mouth on inter-tidal leases were at greatest risk of mortality following summer flood event in February 2004. Therefore to minimise mortalities protective actions such as dropping the baskets, if they are inter-tidal, to lower rungs deeper in the water and higher salinity levels may be considered on, at risk, leases. Further recommended research on freshwater flooding and its effect on farmed oysters include controlled trials on the histopathological impact of sediment on oysters and the changes in the gene expression during low salinity stress.

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